[redacted]

CANADIAN PETROLEUM ASSOCIATION

92-00396/

Prepared for the
Canadian Petroleum Association
and
Environment Canada

By
Nina Novak, P. Eng.
Western Research
Calgary, Alberta

Optimization of Organic Contaminant and Toxicity Testing Analytical Procedures for Estimating the Characteristic and Environmental Significance of Natural Gas Processing Plant Waste Sludges

October 1990

Funding for this project was provided by the CPA Environmental Research Advisory Council (ERAC) and the Federal Panel for Energy Research and Development (PERD).

The CPA has made every effort to assure the accuracy and reliability of the information and data contained in this document. However, the CPA makes no representation, warranty or guarantee as to the legality, reliability or acceptability of the information contained herein and hereby disclaims any liability for any loss or damage arising from the issue of this document or for violation of any federal, provincial or municipal laws, by-laws or regulations. Use of trade names by the authors does not represent endorsement by the CPA.

SUMMARY

The Gas Plant Sludge Characterization: Phase IIB Program is a continuation of the Canadian Petroleum Association (CPA) initiatives to characterize sludges generated at gas processing plants. The study, co-funded by the CPA and Conservation and Protection, Environment Canada, has evolved with the present objectives being to develop, validate and publish a cost-effective methodology for determining the characteristics and environmental significance of natural gas plant sludges.

Phase I of the program, published in 1985 and entitled "Information Review and Development of a Methodology for Determining the Characteristics and Environmental Significance of Natural Gas Processing Plant Waste Sludges" addressed:

- sludge types generated by different gas plant processes;
- subjective ranking of sludge types based on potential environmental concern;
- proposed method for sludge sampling and homogenization; and
- proposed methodologies for inorganic, organic and toxicity testing of gas plant sludges and liquid extracts.

The recommended procedures were incorporated in the assessment of three sludges collected from an Alberta sour gas plant during the Phase II preliminary validation project.

The Phase II report, presented in 1987, focussed on the suitability and reliability of the proposed Phase I methods for characterizing the gas plant sludges. The Phase II study addressed:

- sludge sampling and handling procedures;
- homogeneity of samples;
- accuracy and precision of inorganic and organic measurements;
- quality assurance/quality control requirements; and
- effectiveness of specific toxicity tests in determining environmental significance.

As a result of the Phase II recommendations, it was decided to pursue some of the specific recommendations with further research. Phase IIA concentrated on resolving specific inorganic analytical procedure difficulties and Phase IIB, presented in this report, conducted further study on improving the cost-effectiveness of the organic analysis by evaluating a more comprehensive organics screening step and on developing an effective battery of aquatic and terrestrial toxicity tests.

The specific objectives of the Phase IIB program were:

- To develop an effective procedure for screening waste sludges or centrifugate/leachate generated from sludge samples for volatile, solvent-soluble and water-soluble organics. The screening procedure developed should be straightforward so that non-organic chemists can interpret the results and determine if further detailed characterization is required;

- To verify the reproducibility of the three aquatic toxicity tests recommended as the battery of tests for determining the environmental significance of sludge centrifugates or leachates;

- To assess the performance of two terrestrial toxicity tests in determining the environmental significance of whole sludge samples applied to soil; and

- To assess and discuss the reproducibility and cost-effectiveness of the sampling and analytical techniques proposed for the overall sludge characterization procedure (excluding inorganic analytical techniques).

Phase IIB incorporated a number of the Phase II recommendations and evaluated their practicality and effectiveness. The Phase II sludge sampling and sample generation recommendations generally proved effective and only minor modifications were incorporated during Phase IIB. The organic analysis component of the Phase IIB study developed a sample fractionation and component screening protocol for semi-volatile organics, target volatile organics, specific water-soluble organics and full gas chromatography (GC)/mass spectrometry detection (MS) characterization. The toxicity testing protocol utilized in Phase IIB was derived from the Phase II recommendations. Emphasis was placed on data quality methods and indicators such as toxicity test sample replication, pre-testing meeting and coordination, confidence interval reporting, reference toxicant testing procedure for increased standardization and maximization of communication for procedure clarification. Statistical analysis of the toxicity test results was performed to establish the interlaboratory reproducibility of results.

Sampling was conducted in December 1987 and included sludges from a diethanolamine gas sweetening process dry disposal pit, a sulfinol sweetening process burn pit and a monoethanolamine sweetening process surface water holding pond.

Major conclusions and recommendations from the Phase IIB project are provided in the following sections.

SAMPLE COLLECTION, PREPARATION AND DISTRIBUTION

The sludge sampling procedural, safety and transportation recommendations incorporated from Phase II proved effective for representative sample collection during this study. The pre-sampling site visit by the sampling team is essential in providing familiarity with the site, required equipment, safety precautions and initial sample characterization for sample volume determination. Care must be taken during sample handling, transport and storage to ensure minimal chemical degradation by proper container choice, storage temperature and minimal in-transit time. Samples containing multiple fluid phases must be further evaluated to establish an appropriate method of homogeneous sample generation. The following recommendations are made as a result of this study:

Sampling

- It is recommended that all sludge containment sites be visited prior to sampling to develop appropriate sampling methodology and to obtain a preliminary sample for initial characterization.

- The use of a crane outfitted with a man-basket proved very efficent for the collection of sludge samples from small ponds. Utilizing a two-man sampling crew, a large representative sample (120 L) could be obtained, under good weather conditions, in a single working day.

- A light core sampler, consisting of aluminum or polyethylene tubing and capped with an air-tight seal, proved effective for the collection of most sludges and could be modified with a cork capping for less consolidated sludges.

- Where water depths exceed one to two metres and sludge depths are shallow (less than 0.5 m), a dredge may prove more effective for sample collection than a core sampler.

- The CPA sludge sampler would not retain the sludge sample without the use of a core catcher and is very heavy and cumbersome to use. It did not prove effective for the less consolidated sludges sampled and is not recommended for use unless significantly modified.

Personal Protection

- The quantity of sample required and the depth of the ponds in this study increased potential exposure of sampling personnel through splashing of sludge as it was removed from the core sampler. The use of full-face respirators and Saranex-coated Tyvek suits equipped with hoods is recommended. Care should be taken to tape all seams, e.g., around face masks, boots, gloves and hoods, to minimize the potential for direct skin contact with sludge material.

Sample Preparation and Distribution

- The volume of sample required for this study created problems for sample centrifugation and homogenization. Attempts should be made to reduce the sample quantity as much as possible.

- Protective equipment should be worn during the sample homogenization process.

- Preparation of centrifugate using laboratory-sized centrifuges is not recommended for large volume samples. The process is time-consuming, tedious and exposes laboratory personnel to sludge vapours and liquids over relatively long periods of time. Tests should be undertaken to compare the chemical composition of the aqueous phase of the decanted liquids to that of the centrifugate to determine if this liquid would provide the same information, thereby eliminating the need to centrifuge. However, preparation of smaller volumes of centrifugate (one litre) with laboratory-sized centrifuges for chemical and toxicity test screening would still be cost-effective.

- Testing should be undertaken to determine if, and to what extent,the homogenization and subsampling procedures result in significant losses of volatile organic components.

- All samples must be packaged and shipped to laboratories following TDG Regulations. If samples must be shipped to the United States for analysis, as in this case, additional effort must be expended by shipper and receiver to expedite the timely transport of samples.

- Liquid samples should be assessed to determine whether they are multiphasic. If multiphasic in nature, the central laboratory should consider separating the different phases (e.g., oil and water) prior to external test laboratory distribution. This action is especially relevant in the case of samples for aquatic toxicity testing.

- After preparation and prior to distribution, the accuracy of the reference toxicant test stock solution concentrations should be assessed by chemical analysis and be labelled accordingly. Dilutions of stock solutions should also be analyzed.

ORGANIC ANALYSES

Methods were developed and demonstrated during this project by ENVIRO-TEST LABORATORIES to determine the presence of volatile and semi-volatile pollutants and water-soluble compounds in gas plant sludge and centrifugate samples. Initial Soxhlet extraction was followed by sample fractionation on an alumina column into environmentally significant chemical classes and then analyzed for semi-volatiles. Standard headspace analysis was performed for determination of volatile components. A literature review was undertaken to determine the most effective method for identification of monoethanolamine (MEA) and diethanolamine (DEA) and associated degradation products and a High Performance Liquid Chromatography (HPLC)-derivatization method was selected. The cost-effectiveness of the proposed method exists in the ability to initially screen the semi-volatile fractions with GC/ Flame Ionization Detection (FID), GC/Flame Photometric Detection (FPD) or GC/Nitrogen Phosphorus Detection (NPD) rather than the more expensive GC/MS analysis. In this study, initial screening results were confirmed with GC/MS to determine the effectiveness of the procedure.

Quality control was provided through the use of column calibration compounds to ensure proper column fractionation, and surrogate compounds to determine the appropriate component recovery efficiency for each procedure. One sludge and one centrifugate sample were analyzed by an additional laboratory to provide an indication of procedure reproducibility. Extensive communication between the two laboratories preceded analysis in attempts to reduce a potential misinterpretation of procedural instructions.

The results obtained by ENVIRO-TEST show that the fractionation method effectively divided the samples into chemical subgroups. The alumina column standardization through the use of the calibration compounds is essential both for determination of the appropriate column deactivation level and for quality control by providing an evaluation of other laboratories' abilities to prepare a properly functioning column. This study has shown this to be a critical factor in interlaboratory reproducibility and the results indicate that the problems observed may be minimized by further protocol clarification, as well as establishment of a definitive method for the analysis of multiple phase samples. Problems with interlaboratory comparison of results also occurred as a result of varying methods of compound identifications, e.g., computerized versus manual. This is an interpretation problem rather than the primary objective of technical interlaboratory reproducibility.

The effective chemical separation obtained by ENVIRO-TEST, as presented in Table 1, however, can not clearly identify the source of sample toxicity without additional information for comparison, such as the biological toxicity of the samples.

The following recommendations are made as a consequence of the results of the organic analyses:

- Both the sample fractionating column and the screening analysis must be validated through the use of calibration compounds, as well as surrogates. The results of this phase of the study show some problems with reproducibility, which can be traced to the quality of the fractionation column. The use of calibration compounds would provide a test of the fractionating column's capability of adequate chemical class separation.

Table 1. Summary of Enviro-Test general GC chemical results.

Parameter		Sludge Concentration (µg/g)			Centrifugate Concentration (mg/L)		
		Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
Total Fractions[1]:	Hexane	9,461[2] (3)	9,080 (6)	6,280 (2)	17,190 (7)	LT1 (1)	2,240 (2)
	Benzene	11,484 (4)	4,880 (9)	2,119 (7)	27,745 (7)	LT1 (1)	147 (20)
	Chloroform	2,320 (3)	1,040 (3)	--	1,065 (6)	6 (5)	12 (2)
	Methanol/Water	164 (5)	57 (4)	21 (3)	-	-	-
	Acid	--	-	-	4,181 (9)	4 (5)	81 (2)
Chemical Classes:	Saturates	3,421	-	6,055	8,835	LT1	2,200
	PAH/PASH/PANH	7,657	4,398	1,287	2,472	-	138
	Phenols	-	-	--	142	-	-
	BTEX	4	2	3	1,203	2	229
Water Soluble Compounds (Intractables):	MEA	105	-	10	3	LT1	LT1
	DEA	5,800	22	-	12,624	LT1	LT1
	Sulfinol	92	125	-	1,590	LT1	-
	DEG	261	40	-	1,283	47,658	-

---

[1] Total fraction concentration being the summation of components (numbers of chemical families in brackets) found in ENVIRO-TEST general GC/MS Characterization.

[2] The highest value of the three samples is underlined.

- The specifications of interlaboratory recoveries for deuterated surrogates of greater than 50% are generally achievable. Only ENVIRO-TEST presented relative standard deviations and these generally meet the reproducibility criterion of better than 20%. It is recommended that laboratories provide relative standard deviations for deuterated surrogate results, or some other index of variability, as an indicator of the reproducibility of the procedure.

- To further reinforce the reproducibility of a procedure, it is recommended that three to five laboratories develop a range of acceptable data with respect to surrogate recoveries and the range of specific components to be identified. This may require a further standardization of the procedure, centralization of an adsorbent source to minimize column variation and specification of the method to be used for compound identification, eg. specify library or method of manual interpretation for non-target compounds.

- Another aspect of the reproducibility question is the need for a quality control protocol. This would be a standardized and regimented set of procedures that could generate results that provide a secure basis of comparison to other laboratories.

- To provide sufficient data to enable assessment of risk with respect to classification and handling of waste, a balance must be achieved between the needs for chemical characterization and potential environmental impact. The specific compound GC/MS evaluation provides a chemical profile of the sample but lacks considerably when it comes to interlaboratory reproducibility and economic frugality. Similarly, initial screening of the fractions only permits a rough analytical segregation of general groups of compounds without precise evaluation, but with the possibility of better reproducibility. The decision must be made as to what type of balance is necessary between specific chemical composition and biological toxicity that will provide adequate information to safely deal with classification and/or treatment assessments of complex waste materials.

- The organic analyses should be integrated with the toxicity testing if information relating composition and biological toxicity is to be obtained. Coarse initial tests could be done on the entire sample, both chemical and biological, to help identify the type of specific testing that would be most appropriate. This would be followed by sample fractionation and toxicity tests. A tiered and integrated testing procedure such as this would minimize costs, since only toxic fractions would be further analyzed. This method would also permit identification of the toxicant(s) more clearly and support remediation decisions more effectively.

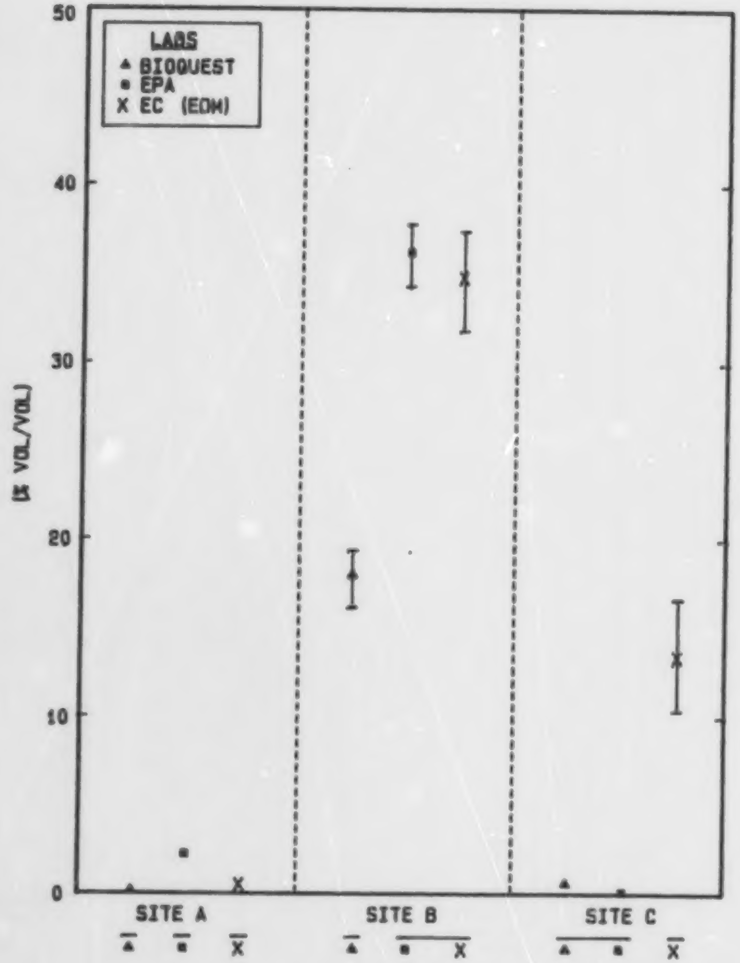
TOXICITY TESTING

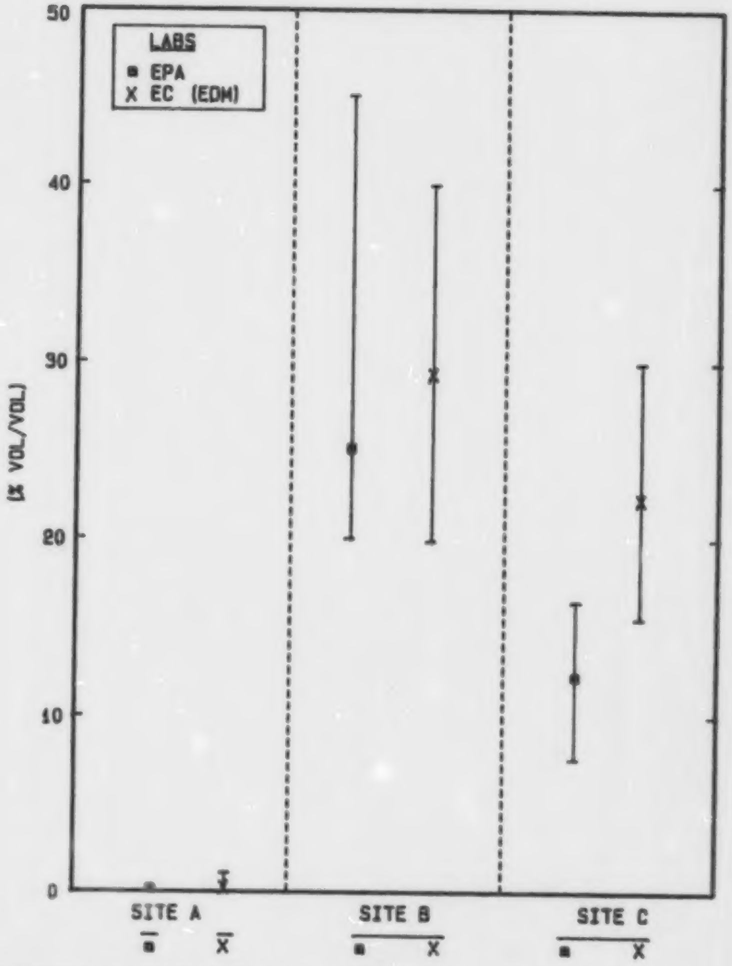
The toxicity testing component of this study evaluated the applicability of three aquatic toxicity tests (Daphnia, algae and Microtox) and two terrestrial toxicity tests (seed germination and earthworm) for estimating the environmental significance of gas plant sludge-generated centrifugate and gas plant sludges, respectively. The organic analyses were performed on the same source samples as those used for the toxicity test to permit valid comparison of results.

The toxicity testing at the participating laboratories was initially checked for comparability with the use of reference toxicants, zinc chloride (an inorganic compound) and 2-chloroacetamide (an organic compound). This testing provided an initial evaluation of the ability of each participating laboratory to conduct each test and an assessment of preliminary interlaboratory comparability. Some significantly different results led to a detailed review of laboratory procedures. Testing of the actual site samples was coordinated to occur with comparable time-frames at the different laboratories, in an attempt to minimize toxicant degradation effects on laboratory reproducibility of results. Extensive efforts were made to communicate common testing procedures to all participants. Tests were performed in triplicate, and means and 95 percent confidence intervals were reported and are presented in Figures 1 through 9.

Detailed statistical analysis of the site test results was performed to evaluate the variability of results both within and among the laboratories, i.e. test the precision of results. Comparison of the 95 percent confidence limits provide a quick and preliminary indication of comparability of results and the confidence in the determined test endpoint. Generally, this comparison agrees with the statistical evaluation of comparability of laboratory results. Variations in the graphical representations are apparent with samples of high toxicity (Sample A) where the statistical evaluation indicates all results are significantly different (due to the small magnitude of the mean and small 95 percent confidence interval), while graphically the results appear similar. Lack of agreement is also observed in some situations where within-laboratory variability (indicated by the coefficient of variation) among participants is substantially different, combined with means that are also noticeably different. In most other cases, the statistically significant differences in inter-laboratory results are also identifiable on the graphical representation of the results. The graphs also present a quick indication of inter-laboratory performance with respect to the precision of their results.

It should be noted that statistically derived within-sample significant differences indicating potential problems with data reproducibility do not interpret directly into biological significance. Laboratories may report statistically different toxicities for a sample but biologically the toxicity may be the





SYMBOLS WITH A COMMON SUPRASCORE ARE NOT SIGNIFICANTLY DIFFERENT, AS DETERMINED BY TUKEY'S HSD 'A POSTERIORI' TEST

FIGURE 1 DAPHNIA MAGNA TOXICITY TEST RESULTS

NOTE: FOR VALUES LESS THAN 5, CONFIDENCE INTERVAL TOO SMALL TO PRESENT ON FIGURES, UNLESS OTHERWISE SHOWN

FIGURE 2 SELENASTRUM CAPRICORNUTUM TOXICITY TEST RESULTS

NOTE: EC (EDM) UTILIZED A DIFFERENT SELENASTRUM TOXICITY TEST PROCEDURE THAN THE EPA LABORATORY

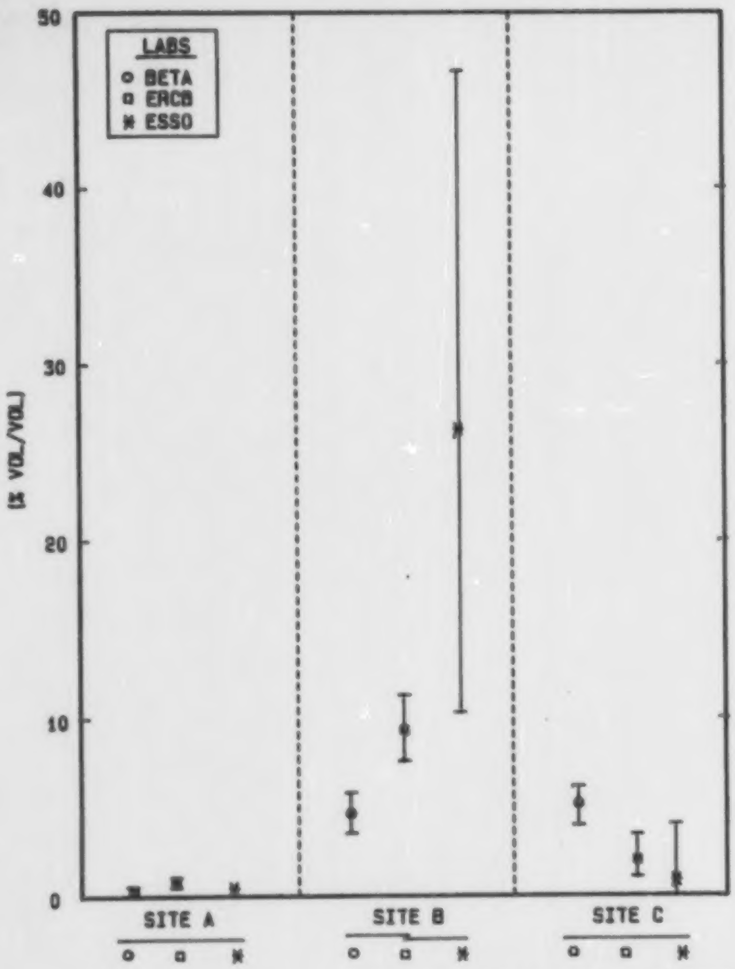


FIGURE 3 MICROTOX EC_{20} (5 MIN) TOXICITY TOXICITY TEST RESULTS

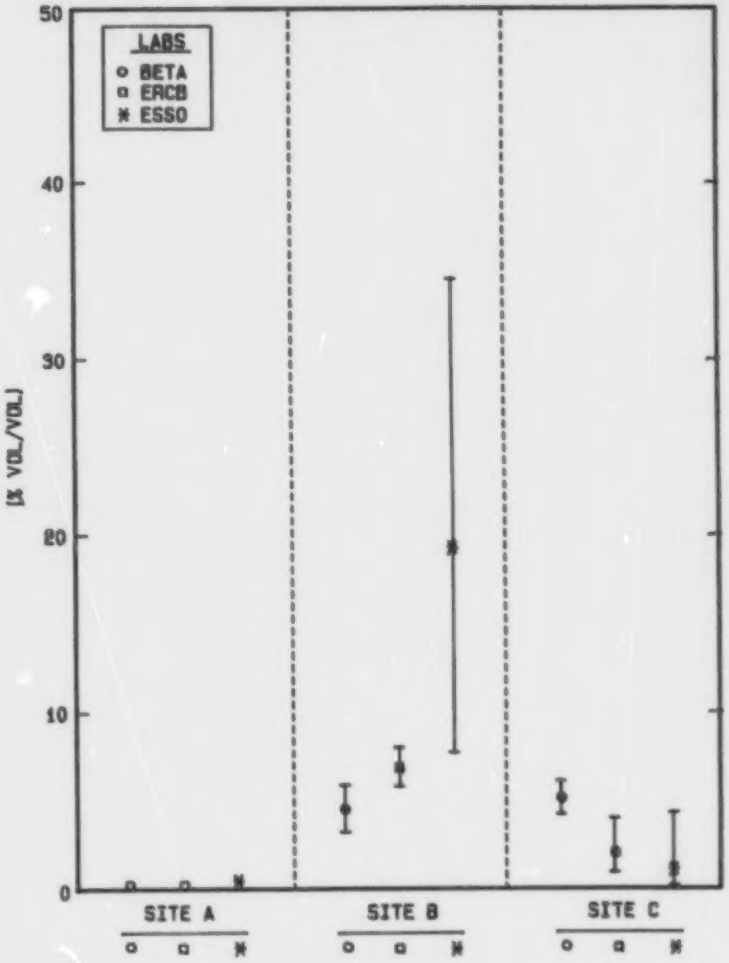
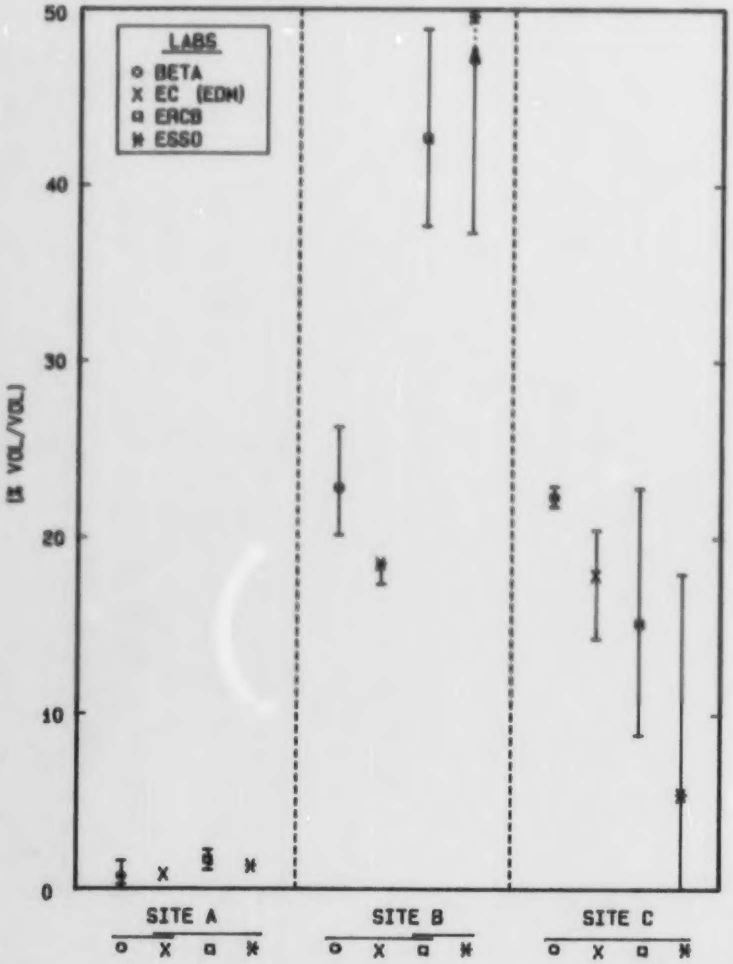
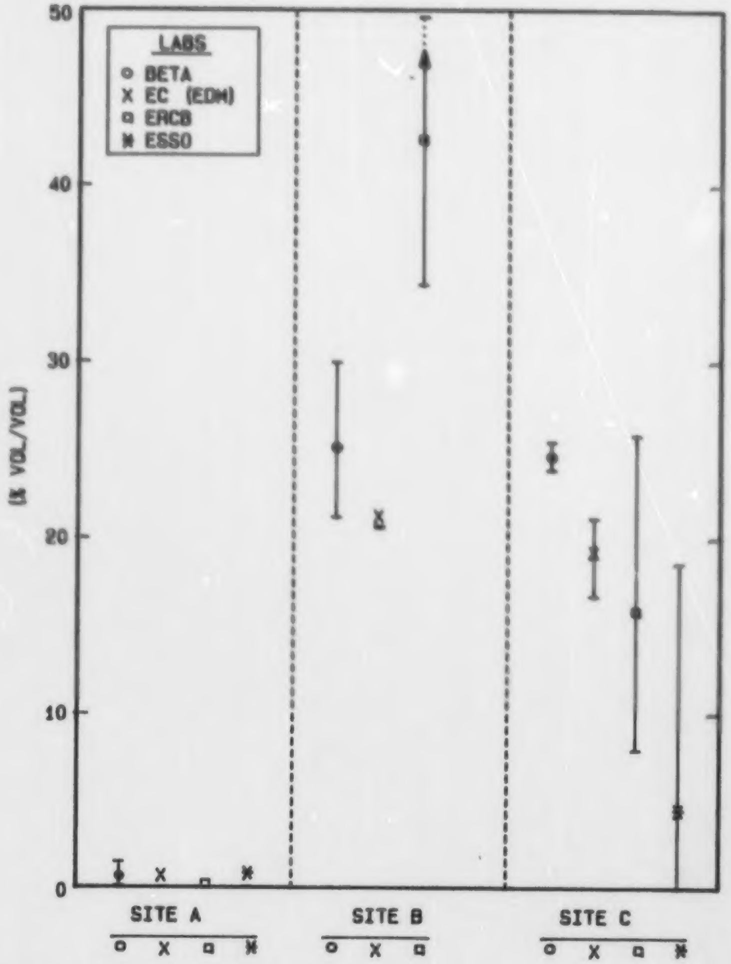


FIGURE 4 MICROTOX EC_{20} (15 MIN) TOXICITY TOXICITY TEST RESULTS

SYMBOLS WITH A COMMON SUPRASCORE ARE NOT SIGNIFICANTLY DIFFERENT, AS DETERMINED BY TUKEY'S HSD 'A POSTERIORI' TEST

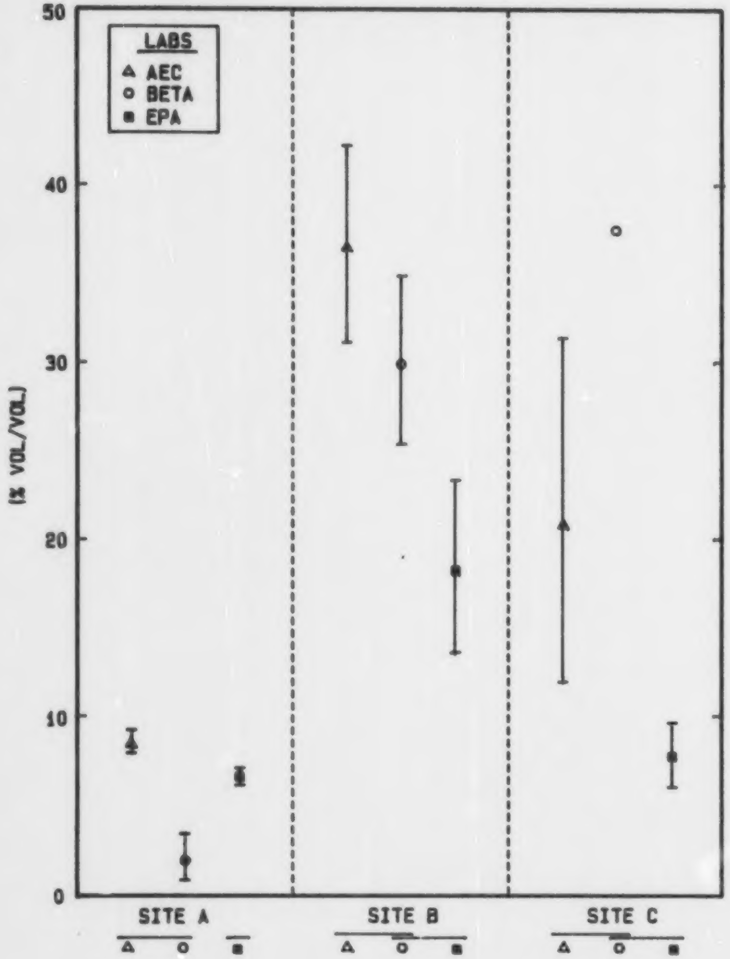


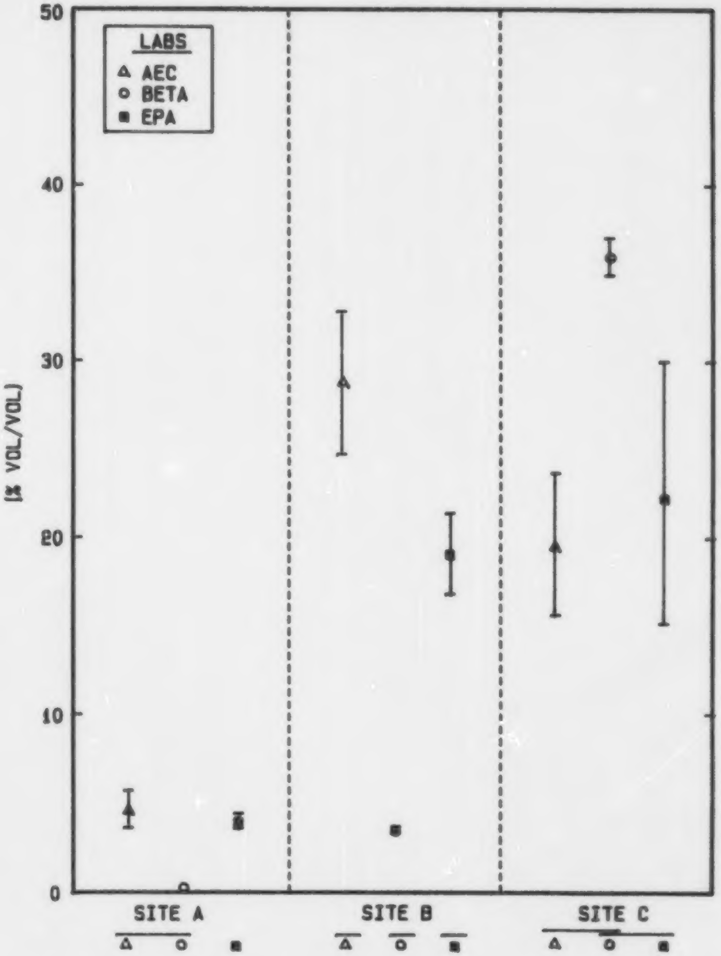


SYMBOLS WITH A COMMON SUPRASCORE ARE NOT SIGNIFICANTLY DIFFERENT, AS DETERMINED BY TUKEY'S HSD 'A POSTERIORI' TEST

FIGURE 5 MICROTOX EC_{50} (5 MIN) TOXICITY TOXICITY TEST RESULTS

FIGURE 6 MICROTOX EC_{50} (15 MIN) TOXICITY TOXICITY TEST RESULTS

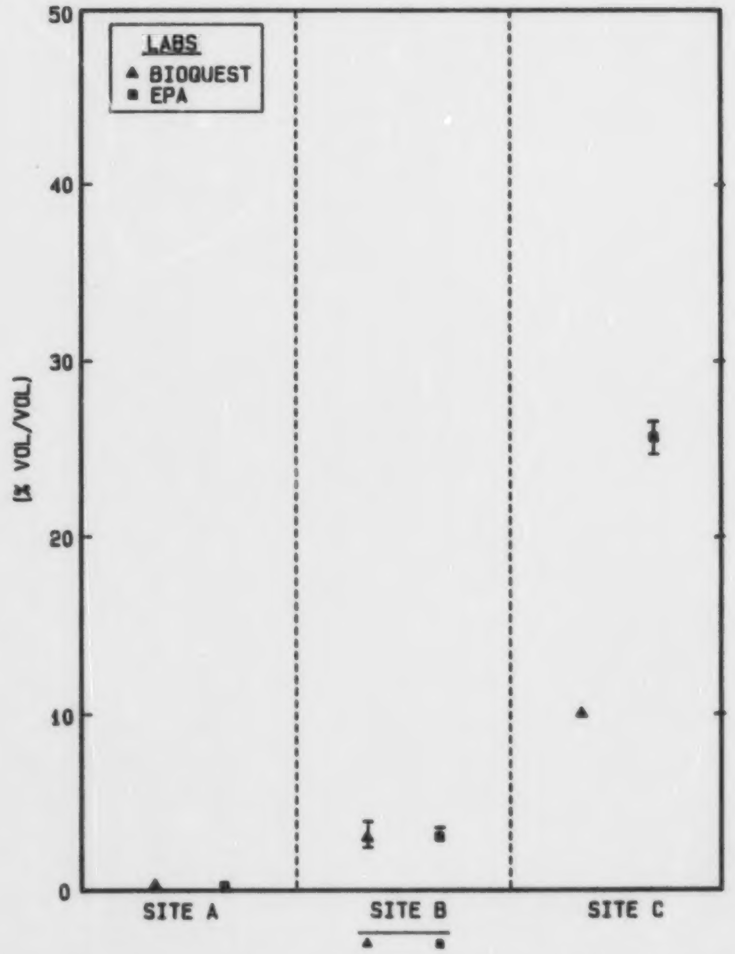




SYMBOLS WITH A COMMON SUPRASCORE ARE NOT SIGNIFICANTLY DIFFERENT, AS DETERMINED BY TUKEY'S HSD 'A POSTERIORI' TEST

FIGURE 7 SEED GERMINATION (CUCUMBER) TOXICITY TEST RESULTS

FIGURE 8 SEED GERMINATION (BARLEY) TOXICITY TEST RESULTS



SYMBOLS WITH A COMMON SUPRASCORE ARE NOT SIGNIFICANTLY DIFFERENT, AS DETERMINED BY TUKEY'S HSD 'A POSTERIORI' TEST

FIGURE 9 EARTHWORM TOXICITY TEST RESULTS

NOTE: VALUES FOR SITE A AND BIOQUEST'S SITE C ARE GIVEN AS RANGES i.e. LESS THAN OR GREATER THAN same. Conversely, some results may be statistically similar to other results based on their large variability, but comparison of the means and confidence intervals indicates there may be different biological impacts. The interpretation of the results should be dictated by the specific concerns applicable to any given sample analysis, i.e. reproducibility of results by laboratories or sample toxicity to a biological system. In this study the focus was on reproducibility of results.

The three aquatic toxicity tests performed comparably well. Both the algae and Daphnia assays generally had within-laboratory coefficients of variation under 20 percent. Even though two different procedures were utilized, the algal assay was the most consistent with respect to within- and among-laboratory variation. Differences were observed in the toxicity endpoints reported using the Microtox test, but the within-laboratory sample variation was still generally less than 40 percent. The EC_{20} derived from the Microtox test presented greater among-laboratory statistical agreement than the EC_{50} results, but this possibly resulted from the observed higher variability of the EC_{20} results, as compared to the EC_{50} tests.

The two terrestrial toxicity tests of the gas plant sludges would likely respond to different toxic components than the aquatic toxicity tests. The trend in toxicity of the various sites tested was reversed for the two less toxic sites (B and C) when compared to the aquatic test trends, as can be seen most clearly in the comparison of the Table 2 earthworm and Microtox results.

Some procedural problems with the earthworm test, resulting from soils water-holding capacity problems, prevented adequate information to be obtained to provide a definitive comparison of test results from the two laboratories. This problem must be resolved if further information is required.

The following recommendations are made as a result of the work conducted during the Phase II B Study:

Table 2. Range of toxicity test mean results.[1]

Toxicity Test[2]	DEA Plant (Sample A)	Sulfinol Plant (Sample B)	MEA Plant (Sample C)
Centrifugate (Aquatic):			
Daphnia (n = 3)	LT[3]0.1-2.3	17.7-36.1	0.2-13.5
Algae (n = 2)	0.2-0.8	25.2-29.5	12.3-22.4
Microtox 15min (n = 3)	0.2-0.9	20.7-42.4	4.5-24.5
Sludge (Terrestrial):			
Cucumber germination (n = 3)	2.1-8.5	18.3-36.7	7.8-37.4
Barley germination (n = 3)	1.0-4.6	3.5-28.6	19.5-36.0
Earthworm (n = 2)	LT 0.6	3.0-3.2	10.0-25.7

---

[1] Greatest sample toxicity values are underlined. Excludes SOS and Toxi-Chromotest results.

[2] EC_{50} or LC_{50} values: n = number of laboratories.

[3] Less than.

SAMPLE DEPENDENT FACTORS

- While considerable effort was expended in attempting to generate homogenous sludge samples, the heterogeneity of the gas plant sludges collected presented some problems. Some samples were oily wastes which separated into different phases upon settling. Actions were taken during this study to counter this problem, i.e. decanting of the oil phase prior to testing. These measures, however, were not adequate to prevent problems and procedures must be established and incorporated into the testing to accommodate this problem. It is recommended that the primary laboratory conduct any pre-testing steps, such as decanting or centrifuging, prior to distribution to an external laboratory for analysis.

- Delays in transportation and testing result in changes in potential toxicants due to various forms of degradation (biological degradation, photolysis, hydrolysis, etc.) leading to different toxicity determinations among laboratories. Adequate preparation and planning must be undertaken in order to prevent this.

- The issue of pH and water hardness adjustment should be addressed in the appropriate test methods. In this study it was decided that, if sample water hardness for the Daphnia test was below 80 mg/L $CaCO_3$, hardness was adjusted up to 200 mg/L since the Daphnia were acclimated to this level. The effect of pH adjustment was briefly investigated and it was apparent that adjustment of pH can cause a change in the detected toxicity level. The degree of effect due to pH adjustment varied among toxicity tests. These preliminary test results indicated that, for a pH change from 8.24 down to 7.04, the EC_{50} value increased approximately 50 percent for Microtox (15 minute). The effect on the algae tested is inconclusive but appears to be reverse to that observed with Microtox.

- When the seed germination test is being considered for evaluation of sludge toxicity and potential disposal alternatives, it is suggested that one of the species to be tested be chosen from representative species in the area of potential contamination. These tests could also report root elongation to monitor developmental toxic plant effects.

PROJECT MANAGEMENT

- The results of this study reinforce the need in future methods validation efforts to place particular attention on test coordination. This would include communication of concise test procedures, compilation of complete test results, standardization of the appropriate method of analysis and evaluation of the results. If the results are unsatisfactory, re-testing requirements would be specified. Communication, as well as the ability to obtain the modifications requested, is of utmost importance.

PROCEDURE STANDARDIZATION

- Increased standardization of toxicity test procedures could be accomplished by further definition of test method variables and determination of the test limits, such as reference toxicant acceptable ranges, which could then be used to assess laboratories' performance with respect to reproducibility and acceptability. The use of five replicates, rather than three, is also recommended for this assessment phase in order to increace statistical confidence in the data. The standardized protocol should also define a source of test organism stock, acclimation period, reference toxicant to be used for test performance appraisal, and information sources when clarification is needed. These are all components of a laboratory's Standard Operating Procedure (SOP). This type of protocol-related information is similar to that which has been prepared recently by Environment Canada for acute lethality testing with Daphnia sp. (Environment Canada, 1990a).

Supplemental testing was performed using two genotoxic tests, the S.O.S. Chromotest and Toxi-Chromotest, on the centrifugate samples. Testing indicated that only centrifugate A presented a genotoxic response. More extensive testing to provide a comparison with other mutagenic tests, such as the Ames test, should be undertaken to identify a reproducible and cost-effective mutagenic test.

A comparison was made with the organic analyses in an attempt to correlate the toxicity and chemical results. The only obvious and consistent relationship observed was that the most toxic sludge samples had the highest summation of chemical component concentrations.

FUTURE CONSIDERATION

The following recommendations should be considered in any future sludge characterization/environmental significance method development research:

- An integrated, tiered protocol for combined organic, inorganic and toxicity test analyses should be attempted to help identify sources of toxicity in a cost-effective manner and to more effectively connect these complimentary types of analyses. This protocol would consist of initial chemical and toxicity testing which would provide information on whether the sample is toxic. Further analysis would occur on fractions generated with the intent to identify the environmentally important chemical classes which are responsible for the observed acute toxicity. This analysis would be of an intermediate level with respect to cost and specific information provided. Detailed chemical analysis, even though more costly, would be performed only if inadequate information existed to that point. This integrated method would permit cost-effective identification of the type of biological toxicity and the chemicals responsible for the observed toxic effects.

This is valuable and essential information when evaluating the environmental impact of a complex waste and when considering methods of remediation and disposal.

- Reference toxicant data should be statistically evaluated to estabish the effect of procedural and analytical variations.

- In the future, a battery of aquatic and terrestrial toxicity tests could provide valuable information on the feasibility of certain types of disposal or remediation methods. For example, an area considered for landfill having a relatively high water table or close to a potable water source would not likely be the best disposal option for a sludge exhibiting high toxicity levels during aquatic toxicity testing. Where land treatment of waste is considered as a disposal option, the seed germination test can select native varieties from a proposed disposal option area to quickly determine their specific sensitivities to potential contaminants in a given sludge.

- This study concentrated on acute lethal and sublethal responses since the tests are quick to perform, with a well defined end-point. These tests provide important information about waste characterization. However, it is recommended that the final battery of tests include at least one genotoxicity test, such as the Ames test or S.O.S. Chromotest, which is capable of identifying potential mutagenic or carcinogenic effects of a waste. If this is pursued, comparison testing between the Ames test, S.O.S. Chromotest and the new Microbics genotoxicity screening procedure on a variety of wastes could assist in choosing the most reproducible, informative, and cost-effective test.

In conclusion, an effective sample collection program was performed, organic and toxicity testing was completed, and this report presents the results of these evaluations. The Phase IIB study has developed an effective procedure for screening waste sludges or centrifugate/leachate generated from sludge samples for volatile, solvent-soluble and water-soluble organics.

The three aquatic and two terrestrial toxicity tests evaluated proved effective in indicating the toxicity of complex mixtures, such as the three gas plant centrifugates and sludges tested. Some variations in precision were experienced with all the toxicity tests. This variation is magnified when the test procedure was not closely adhered to or when sample heterogeneity is encountered. The biological testing variation was, however, no greater than the variation observed with the organic analyses.

The decision as to which tests provide the most appropriate combination for sludge characterization is dependent on the type of treatment and disposal proposed for the waste.

TABLE OF CONTENTS

		Page
EXECUTIVE SUMMARY		i
LIST OF TABLES		xxviii
LIST OF FIGURES		xxx
ACKNOWLEDGEMENTS		xxxi
1.	INTRODUCTION	1
1.1	Background	1
1.1.1	Phase II - Sample Collection and Homogenization	3
1.1.2	Phase II - Inorganic Analyses	3
1.1.3	Phase II - Organic Analyses	5
1.1.4	Phase II - Toxicity Testing	6
1.2	Phase II A & Phase II B - Study Objectives	7
1.3	Phase II B - Scope of Work	8
1.4	Project Chronology - Phase II B	12
2. SLUDGE SAMPLING		14
2.1	Sample Requirement	15
2.2	Sample Locations	17
2.2.1	Plant A	17
2.2.2	Plant B	17
2.2.3	Plant C	18
2.3	Sample Collection	18
2.3.1	Health and Safety	18
2.3.2	Sampling Procedures	19
2.3.3	Sample Handling in the Field	23
2.3.4	Sample Transport and Storage	23
2.3.5	Sample Homogenization and Splitting	24
2.3.6	Sludge Centrifugation	25
2.3.7	Centrifugate Homogenization and Sub-Sampling	26
2.3.8	Sample Packaging and Distribution	26
2.3.9	Reference Toxicant Preparation and Distribution	27
2.3.10	Pre-Testing Treatment of Multiphasic Centrifugate Samples	28
3.	ORGANIC ANALYSES	29
3.1	Literature Survey	29
3.2	Summary of Organic Contaminant Analytical Program	31
3.2.1	Sample Preparation, Extraction and Analyses-Sludges	31
3.2.1.1	Sludge Analysis for Semi-Volatile Priority Pollutants	32
3.2.1.2	Sludge Analysis for Volatile Priority Pollutants	37
3.2.1.3	Sludge Analysis for Water Soluble Compounds	37

TABLE OF CONTENTS (continued)

Page

3.2.1.4 Extraction of Sludge Samples for Alkanolamine Degradation Products 39
3.2.1.5 Quality Control for Alkanolamine Degradation Products 39
3.2.1.6 Derivatization of Centrifugates and Sludge Extracts For Analysis of Alkanolamine Degradation Products 40
3.2.2 Sample Preparation, Extraction and Analysis - Centrifugates 41
3.2.2.1 Centrifugate Analysis for Semi-Volatile Priority Pollutants 41
3.2.2.2 Centrifugate Analysis for Volatile Priority Pollutants 42
3.2.2.3 Centrifugate Analysis for Water Soluble Compounds 42
3.2.2.4 Analysis of Centrifugates for Alkanolamine Degradation Products 42
3.2.3 Analyses of Sludge and Centrifugates for Conventional Inorganic Parameters 42
3.2.4 Chromatographic Conditions 42
3.2.4.1 Gas Chromatography/Flame Ionization Detection for Semi-Volatile Priority Pollutants 42
3.2.4.2 Gas Chromatography/Flame Ionization Detection for Volatile Priority Pollutants 43
3.2.4.3 Gas Chromatography/Flame Photometric Detection for Sulfinol 43
3.2.4.4 Gas Chromatography/Mass Spectrometry Detection for Diethylene Glycol 43
3.2.4.5 High Pressure Liquid Chromatography Detection for Monoethanolamine and Diethanolamine 44
3.2.4.6 Detection Limits 44
3.3 Inter-Laboratory Method Differences 44
3.4 Results 47
3.4.1 Quality Control for Sludge Sample Analyses . . . 47
3.4.1.1 Validation of Alumina Column 47
3.4.1.2 Recovery of Surrogates from Sludge 49
3.4.1.3 Recovery of Water-Soluble Compounds from Fortified Sludge 51
3.4.1.4 Validation of Method for Alkanolamine Degradation Products 51
3.4.2 Surrogate Recovery in Centrifugate Sample Analyses 54
3.4.3 Sludge Analyses Results 54
3.4.3.1 Results of Sludge Analyses for Semi-Volatile Priority Pollutants 54
3.4.3.2 Results of Sludge Analyses for Volatile Priority Pollutants 56
3.4.3.3 Results of Sludge Analyses for Water Soluble Compounds 56
3.4.3.4 Analysis of Sludge for Conventional Parameters 56

TABLE OF CONTENTS (continued)

Page

3.4.3.5 Alkanolamine Degradation Products Results Using HPLC/Fluorescence 56
3.4.3.6 Alkanolamine Degradation Products Results Using GC/FID 59
3.4.3.7 Characterization of Sludges using Gas Chromatography/Mass Spectrometry 59
3.4.4 Centrifugate Analyses Results 60
3.4.4.1 Results of Centrifugate Analyses for Semi-Volatile Priority Pollutants 60
3.4.4.2 Results of Centrifugate Analyses for Volatile Priority Pollutants 60
3.4.4.3 Results of Centrifugate Analyses for Water Soluble Compounds 60
3.4.4.4 Analysis of Centrifugate for Conventional Parameters 69
3.4.4.5 Characterization of Centrifugates using Gas Chromatography/Mass Spectrometry 69
3.5 Discussion of Results 69
3.5.1 Comparison of ENVIRO-TEST Sludge and Centrifugate Results 69
3.5.2 Evaluation of Volatile and Semi-Volatile Pollutant Analysis Method Performance 79
3.5.3 Performance of the Water-Soluble Analysis Method 81
3.5.4 Alkanolamine Degradation Products Evaluation . . 81
3.5.5 Inter-Laboratory Variability 82
3.5.6 Cost Effectiveness of Proposed Organic Chemical Fractionation Method 83

4. TOXICITY TESTING. 85
4.1 Procedure Standardization using Reference Toxicants 88
4.2 General Method of Statistical Analysis of Results 91
4.2.1 Within-Laboratory Reproducibility 92
4.2.2 Among-Laboratory Variation 93
4.3 Daphnia Toxicity Test 94
4.3.1 Test Procedure 94
4.3.2 Results 95
4.3.3 Statistical Assessment of Daphnia Test Reproducibility 95
4.4 Algal Toxicity Test 98
4.4.1 Test Procedure 98
4.4.2 Results 99
4.4.3 Statistical Assessment of Algal Test Reproducibility 99
4.5 Microtox Toxicity Test 102
4.5.1 Test Procedure 102
4.5.2 Results 103
4.5.3 Statistical Assessment of Microtox Test Reproducibility 106

TABLE OF CONTENTS, (Continued)

page

4.6 Seed Germination Toxicity Test 113
4.6.1 Test Procedure 113
4.6.2 Results 114
4.6.3 Statistical Assessment of Seed Germination Test Reproducibility 114
4.7 Earthworm Toxicity Test 117
4.7.1 Test Procedure 117
4.7.2 Results 120
4.7.3 Statistical Assessment of Earthworn Test Reproducibility 120
4.8 S.O.S. Chromotest 123
4.8.1 Test Procedure 123
4.8.2 Results 123
4.9 Toxi-Chromotest 124
4.9.1 Test Procedure 124
4.9.2 Results and Comparison to Microtox Response . . 124
4.10 Effect of pH Adjustment 126
4.11 Comparison of Toxicity Test Results 128
4.12 Discussion of Potential Toxicant Sources in Sludge Samples 130
4.13 Interpretation of Toxicity Test Results 131
4.14 Quality Assurance in Toxicity Testing 136
4.15 Cost-Effectiveness of Various Toxicity Tests . 139

5. CONCLUSIONS AND RECOMMENDATIONS 141
5.1 Conclusions 141
5.2 Recommendations 145
5.2.1 Sampling and Distribution Recommendations . . . 145
5.2.2 Organic Analyses Recommendations 148
5.2.3 Toxicity Testing Recommendations 149

6. ACRONYMS 154

7. REFERENCES 156

APPENDICES

Appendix A: Sludge Sampling Plan 1A

Appendix B: Toxicity Test Procedures 1B

Appendix C: Raw Toxicity Test Data and Confidence Limits 1C

LIST OF TABLES

Page
1.1 Phase IIB Toxicity Testing Program 10

2.1 Sludge and Liquid Centrifugate Requirements 16

3.1 Constituents of Surrogate Mix 34
3.2 Constituents of Adsorbent Column Calibration Mixture . 35
3.3 EPA Target Compound Priority Pollutant Compounds Analyzed by GC/MS at ENVIRO-TEST - EPA Method 625 and Method Detection Limits 45
3.4 Recoveries of Calibration Compounds from Alumina Column 48
3.5 Surrogate Recovery from Sludges 50
3.6 Recoveries of Water Soluble Compounds from Sludge . . . 52
3.7 Recoveries of Alkanolamine Degradation Products from Fortified Sample C 53
3.8 Surrogate Recovery from Centrifugates by ENVIRO-TEST . 55
3.9 Analysis of Sludges for Semi-Volatile Priority Pollutants 55
3.10 BTEX Analysis of Sludges 57
3.11 Sludge Analysis for Water Soluble Target Compounds . . 57
3.12 Conventional Parameters of Sludge Samples 58
3.13 General GC/MS Characterization of Site A Sludge 61
3.14 General GC/MS Characterization of Site B Sludge 62
3.15 General GC/MS Characterization of Site C Sludge 63
3.16 GC/MS Specific Compound Identification for Site A Sludge 64
3.17 GC/MS Specific Compound Identification for Site B Sludge 65
3.18 GC/MS Specific Compound Identification for Site C Sludge 66
3.19 Analysis of Centrifugate Samples for Semi-Volatile Priority Pollutants 67
3.20 BTEX Analysis of Centrifugates 68
3.21 Centrifugate Analysis for Water Soluble Target Compounds 68
3.22 Conventional Parameters for Centrifugate Samples . . . 70
3.23 General GC/MS Characterization of Site A Centrifugate . 71
3.24 General GC/MS Characterization of Site B Centrifugate . 72
3.25 General GC/MS Characterization of Site C Centrifugate . 73
3.26 GC/MS Specific Compound Identification for Site A Centrifugate 74
3.27 GC/MS Specific Compound Identification for Site B Centrifugate 75
3.28 GC/MS Specific Compound Identification for Site C Centrifugate 76
3.29 Summary of ENVIRO-TEST General GC Chemical Results . . 78

4.1 Aquatic Toxicity Test Reference Toxicant Results. . . . 89
4.2 Terrestrial Toxicity Test Reference Toxicant Results . 90
4.3 Summary Statistics for *Daphnia magna* LC_{50} Toxicity Tests 96

LIST OF TABLES (continued)

Page

4.4 Coefficients of Variation for *Daphnia magna* LC_{50} Toxicity Tests 97
4.5 Summary of ANOVA analysis in *Daphnia magna* LC_{50} Estimates 97
4.6 Summary Statistics for *Selenastrum capricornutum* EC_{50} Toxicity Tests 100
4.7 Coefficients of Variation for Algae EC_{50} Toxicity Tests 100
4.8 Summary of One-Sample t-Test for Algae Toxicity Tests . 101
4.9 Summary Statistics for Microtox EC_{20} Toxicity Tests 104
4.10 Summary Statistics for Microtox EC_{50} Toxicity Tests 105
4.11 Coefficients of Variation for Microtox EC_{20} Toxicity Tests 107
4.12 Coefficients of Variation for Microtox EC_{50} Toxicity Tests 108
4.13 Summary of ANOVA Analysis for Microtox EC_{20} Toxicity Tests 110
4.14 Summary of ANOVA Analysis for Microtox EC_{50} Toxicity Tests 111
4.15 Results Summary for Seed Germination Toxicity Tests . . 115
4.16 Coefficients of Variation for Seed Germination Toxicity Tests 116
4.17 Summary of ANOVA Analysis for Toxicity Tests for Seed Germination 118
4.18 Summary Statistics for *Eisenia andrei* LC_{50} Toxicity Tests 121
4.19 Coefficients of Variation for *Eisenia andrei* Toxicity Tests 122
4.20 Summary of t-Test Analysis for Toxicity Tests for *Eisenia andrei* EC_{50} 122
4.21 Toxi-Chromotest Bacterial Assay Results 125
4.22 pH Adjustment Effect on Microtox and Algae Results. . . 127
4.23 Range of Centrifugate (Aquatic) Toxicity Test Mean Results 129
4.24 Range of Sludge in Soil (Terrestrial) Toxicity Test Mean Results 129
4.25 Comparative Toxicity of Centrifugate Samples 133
4.26 Comparative Toxicity of Sludge Samples 134
4.27 Individual Toxicity Testing Costs for Phase IIB 140
4.28 Canadian Private Sector Toxicity Testing Costs (1987$) 140

LIST OF FIGURES

Page

2.1 Sludge Coring System 20

3.1 Analysis of Gas Plant Sludge for Semi-Volatile Priority Pollutants 33

3.2 Analysis of Gas Plant Sludge for Intractable Compounds 38

ACKNOWLEDGEMENTS

Western Research wishes to acknowledge the assistance provided by the Study Review Committee throughout this study. Members of the Study Review Committee were:

- B.R. Croft, Chairman, Study Review Committee, Amoco Canada Petroleum Company Ltd.;
- R.P. Scroggins, Conservation and Protection, Environment Canada;
- L.T.L. Callow, Gulf Canada Resources Limited;
- K. Johnson, Shell Canada Resources Limited;
- M. Burley and K. Moynihan, Esso Resources Canada Limited; and
- M. Korchinski, Alberta Energy Resources Conservation Board.
- J. Fujikawa, Alberta Environment

Western Research also acknowledges the services provided by its subcontractors in this study and by the toxicity testing laboratories. Specific thanks are accorded to Diebe Birkholz of Enviro-Test Laboratories and Eric Peake of the Kananaskis Centre for Environmental Research for their contribution in evaluating procedures and completing major sections of the report.

Funding for this study was provided jointly by the Environmental Research Advisory Council of the Canadian Petroleum Association and the Federal Panel on Energy Resources and Development (PERD).

1. INTRODUCTION

The Gas Plant Sludge Characterization: Phase IIB Program is a continuation of the Canadian Petroleum Association (CPA) initiatives to characterize and estimate the environmental significance of waste sludges generated at gas processing plants.

This research is jointly funded by the CPA and Conservation and Protection, Environment Canada. This method development research effort is in its fourth year. The Phase II A and II B studies were preceded by a Phase I (Monenco, 1985) proposed methodology study and the first method validation study, Phase II (Monenco, 1987).

1.1 BACKGROUND

A significant number of Western Canada gas plants are scheduled for decommissioning over the next 20 years. Therefore, the Canadian Petroleum Association's Environmental Research Advisory Council approached Environment Canada to co-fund a multi-year research program to characterize the residual waste sludges. The program has evolved with the present objectives being to develop, validate and publish a cost-effective methodology for determining the characteristics and environmental significance of natural gas plant sludges.

In March 1984, an industry/government Technical Steering Committee was formed to direct this multi-year research program. Agencies represented on the technical committee include:

- Environment Canada
- Alberta Department of the Environment
- Alberta Energy Resources Conservation Board
- Dome Petroleum Ltd. (now Amoco Petroleum Canada Co. Ltd.)
- Gulf Canada Resources Limited
- Esso Resources Canada Limited
- Shell Canada Resources Limited

In 1985, the Canadian Petroleum Association published a final report for Phase I of the program entitled "Information Review and Development of a Methodology for Determining the Characteristics and Environmental Significance of Natural Gas Processing Plant Waste Sludges". The Phase I report:

- outlined sludge types generated by different gas plant processes;
- provided a subjective ranking of sludge types based on potential environmental concern;
- proposed a method for sludge sampling and homogenization; and
- proposed methodologies for inorganic, organic and toxicity testing of gas plant sludges and liquid extracts.

The recommended procedures outlined in the Phase I report were used in the assessment of three sludges collected from an Alberta sour gas plant during the Phase II preliminary field validation project. The main objective of the Phase II project was to focus on the suitability and reliability of the proposed methods for characterizing and determining the environmental significance of gas plant sludges. To meet these objectives, the project addressed:

- sludge sampling and handling procedures;
- homogeneity of samples;
- accuracy and precision of inorganic and organic measurements;
- quality assurance/quality control requirements; and
- effectiveness of specific toxicity tests in determining environmental significance.

A final report on the findings of the Phase II preliminary field validation project was published by the Canadian Petroleum Association in March 1987. A number of recommendations were outlined in this report as to how inorganic, organic and toxicity testing assessment procedures could be improved and made more cost-effective.

To ensure that the best possible procedure for assessing gas plant sludges was developed, the Technical Steering Committee decided to pursue, through further research, some of the recommendations outlined in the Phase II report. The continued research has proceeded as two separate but parallel projects, Phase IIA and IIB. The Phase IIA project concentrates on resolving specific inorganic analytical procedure difficulties identified by the Phase II work. In the Phase IIB project, further research was conducted to improve the cost-effectiveness of the organic analysis screening step and to develop an effective battery of aquatic and terrestrial toxicity tests.

Based on the findings of the Phase I, II, IIA, and IIB studies, a final report will be prepared which will outline the methodology recommended for sampling, chemically characterizing and estimating the environmental significance of gas plant waste sludges.

1.1.1 Phase II - Sample Collection and Homogenization

The basic sampling techniques and sample handling procedures conducted in the Phase II study were adequate. The study recommended that sampling concentrate on sludge from waste pits or ponds, as it was felt that these sources were more pertinent environmental management concerns at gas processing plants.

Sample collection and management could also be improved by conducting a pre-sampling site reconnaissance and employing an experienced sampling team. It was recommended that a composite sample be generated from a large number of subsamples (a minimum of six) to produce a single representative sludge sample from each source. It was also recommended that the sample volume be reduced as much as possible to minimize sample management concerns.

1.1.2 Phase II - Inorganic Analyses

During the Phase II study, inorganic analyses led to difficulties in confirming sludge composition, particularly when results were compared between laboratories. Reasonably good reproducibility was observed for the following parameters:

- loss on drying (moisture content);
- pH and electrical conductivity;
- oil;
- total nitrogen; and
- metals (As, Cd, Co, Cu, Fe, Mn, Ni, Va, Zn).

Results for the following parameters were reproducible between laboratories, however, inconsistencies reduced levels of confidence to within a factor of 2 (ie. 100%):

- ammonia;
- total sulphur and total carbon;
- nitrate;
- phenols; and
- metals (Cr, Hg, Mo, Se).

Poor reproducibility of results was observed for the following parameters:

- cyanide; and
- metals (Al, Ba, Bo, Pb, Th).

It was recommended that further research be conducted to establish reliable procedures for these inorganic parameters, although it was recognized that these analytical problems were likely common to the measurement of inorganic parameters in any complex oily waste sludge type.

For the above problem parameters, it was recommended that analytical procedures be documented, confidence limits be established and statistical evaluation of replicate analyses be conducted, representative standards be prepared and that an inter-laboratory audit program be initiated.

Measurement of inorganic parameters in liquid centrifugates and leachates during the Phase II project did not seem to be a problem.

1.1.3 Phase II - Organic Analyses

During Phase II, organic analyses were conducted on extracted sludge samples, centrifugates and leachates. Organic compounds were extracted from sludge samples by Soxhlet extraction. Results were validated by use of deuterated compounds and duplicate analysis by a quality control laboratory. Analysis was conducted by GC/MS for both EPA Priority Pollutants (quantitative analysis for target compounds, solvent extractables only) and non-target compounds (semi-quantitative analysis).

A few EPA Priority Pollutants were identified in sludge samples at low concentrations. These included naphthalene and phenanthrene/ anthracene, which likely originate in the raw gas, and chlorophenols which may be associated with wood preservatives.

Results differed somewhat between laboratories and some compounds were identified by one laboratory and not by the other. However, these differences could have been a result of several factors, including low compound concentrations (i.e., at or near detection levels), sample homogeneity, sample pH (adjusted at one laboratory), differences in interpretation of mass spectra, and high water solubility (hydrophilic nature) with low solvent extractability.

The following recommendations came out of the Phase II investigation:

- sludge samples should be neutralized prior to extraction to improve recovery;

- samples should be screened for organic carbon to determine analytical sample size;

- the amount of alumina used for column chromatography should be increased; and

- analysis should be conducted in stages by employing GC/FID analysis on fractions prior to GC/MS analysis.

The centrifugate and leachate samples were also analyzed for organic compounds. Some compounds that were not observed in the original sludge samples were detected and, conversely, some compounds identified in sludge samples were not found in the liquid fraction. It was concluded that water solubility differences were the major factor involved here.

Alkanolamines and specific degradation products were analyzed in the samples. Test methods used (detection limits of 100 µg/g) were suitable for identifying MEA and degradation products.

1.1.4 Phase II - Toxicity Testing

Toxicity testing in the pilot program (Phase II) included the seed germination, algae, rainbow trout, *Daphnia*, Microtox and nematode toxicity tests. Tests were conducted on liquid fractions generated from both centrifuging and leaching sludge samples.

Results were generally consistent and reproducible; however, inter-laboratory variability of results occurred. The Phase II study concluded that high inter-laboratory variability was due to: 1) discrepancy likely resulting from changes in sample toxicity during storage, and 2) different testing initiation dates among the participating laboratories.

The Phase II report recommended that future toxicity testing be conducted, preferably on "centrifugate" samples. Testing of lab-generated leachates would be conducted in cases where sufficient centrifugate was unavailable. It was also recommended that 95 percent confidence limits be calculated for all tests. Better quality control was recognized as being required in preparing, handling and shipping samples for toxicity testing, and it was noted that in cases of significant toxic response, follow-up chemical analysis should be considered to identify potential toxic compounds. Results of the Phase II study suggest that pH and the presence of alkanolamines at high levels contributed significantly to the toxicity of samples.

1.2 PHASE IIA and IIB - STUDY OBJECTIVES

As a response to the recommendations from Phase II, further study was conducted during Phases IIA and IIB. The Phase IIA study objective is to design a method for the total elemental analysis for Ag, As, B, Ba, Be, Cd, Co, Cr, Cu, Hg, Mo, Ni, Pb, Sb, Se, Th, V, and Zn, as well as for cyanide (HCN), in gas processing plant sludges. The study will include literature searches, discussion meetings with qualified individuals to evaluate the available methods, laboratory evaluations of the preferred methods and round-robin-type evaluations of samples collected from the gas plants also studied in Phase IIB.

This study, Phase IIB, incorporated and investigated many of the sludge characterization method recommendations generated by the Phase II Pilot Testing Project. As one component of this multi-year method development/validation program, the specific objectives of the Phase IIB program were:

- To develop an effective procedure for screening waste sludges or centrifugate/leachate generated from sludge samples for volatile, solvent-soluble and water-soluble organics. The screening procedure developed should be straightforward so that non-organic chemists can interpret the results and determine if further detailed characterization is required;

- To verify the precision and reproducibility of the three aquatic toxicity tests recommended as the battery of tests for estimating the environmental significance of sludge centrifugates or leachates;

- To assess the performance of two terrestrial toxicity tests in determining the environmental significance of whole sludge samples applied to soil; and

- To assess and discuss the reproducibility and cost-effectiveness of the sampling and analytical techniques proposed for the overall sludge characterization procedure (excluding inorganic analytical techniques).

1.3 PHASE IIB - SCOPE OF WORK

The Phase IIB project scope of work included the management of all project functions, sludge sampling, organic analysis, toxicity testing, data interpretation and reporting components. Specific project requirements are summarized below.

Sludge Sampling

- Composite sludge samples were to be collected from process wastewater ponds at three gas processing plants. Samples were to be representative of different gas sweetening processes, namely MEA, DEA and sulfinol (the common name of sulpholane).

- A large number of subsamples was requested to generate representative samples, and samples had to be large enough to meet the program requirements for both the Phase IIA and the Phase IIB programs.

- A preliminary reconnaissance trip was to be made to each facility prior to sampling to determine equipment requirements, take samples to determine water content for sample quality determination, and to familiarize the sampling team with the facility and safety procedures necessary.

- Samples for organic analysis and toxicity testing (Phase IIB program) were to be collected, composited and stored in pre-rinsed steel alloy 20 L containers.

- Samples for inorganic analysis (Phase IIA program) were to be collected, composited and stored in 20 L high-density polyethylene containers.

- All samples were to be kept cool (approximately 4°C) throughout the transportation and storage phases.

- Sample preparation for Phase IIB sludge samples required free water decanting, sample homogenization, raw sludge sample splitting, sample centrifugation, centrifugate sample splitting, sample packaging, labeling and distribution to participating laboratories.

Organic Analysis

The participating organic analytical laboratories were pre-selected by the Technical Steering Committee and were Enviro-Test Laboratories, Edmonton, and Kananaskis Centre for Environmental Research (KCER), Calgary. The analytical program included a literature review of applicable analytical methods and development of sludge screening methods. The following analyses were required:

- screening for semi-volatile organics;
- screening for target volatile organics;
- analysis for specific water soluble organic compounds; and
- full GC/MS characterization.

The scope of work included methods development for and analysis of specific amine degradation products, analysis and reporting of results. KCER evaluated one sludge and one centrifugate sample using methods developed by Enviro-Test.

Toxicity Testing

A wide range of toxicity tests were requested as outlined in Table 1.1. All laboratories were to agree on the analytical protocol to be followed prior to commencement of the analyses. Again, all toxicity testing laboratories were pre-selected by the Technical Steering Committee. All tests were to be conducted in triplicate with the timing of toxicity test initiation controlled by the overall project manager.

Table 1.1. Phase IIB - Toxicity testing program.

Toxicity Tests	Laboratories	Number of Samples Per Lab	Number of Analyses Per Lab
AQUATIC			
Daphnia magna lethality/immobility (48 hr LC_{50})	BIOQUEST, EC (Edm), EPA	5[1]	15[3]
Selenastrum captricornutum growth inhibition (96 hr EC_{50})	EC (Edm), EPA	5[1]	15[3]
Photobacterium phosphoreum luminescence inhibition (Microtox, 5 and 15 min. EC_{50}'s)	BETA, ERCB, ESSO, EC (Edm)	5[1]	15[3]
TERRESTRIAL			
Seed germination/plant growth success soil test (2 plant species)	BETA, AEC, EPA	5[2]	126[4]
Eisenia andrei earthworm/soil survival (14 day LC_{50})	BIOQUEST, EPA	5[2]	15[5]
SOS Chromotest	EC (Long), EPA	5[2]	15[5]
Toxi-Chromotest	EPA	5[2]	15[5]

[1] Samples include centrifugate from three sludge samples plus two reference samples.

[2] Samples include three sludge samples plus two reference toxicant samples.

[3] Includes analysis of three samples in triplicate plus two control samples (in triplicate).

[4] Includes analysis of six different concentration on three samples in triplicate for two species of seeds on each sample plus control sample analysis.

[5] Includes triplicate analysis on each sample.

BIOQUEST - Bioquest International, Winnipeg, Manitoba
BETA - Beta Research, Calgary, Alberta
EC (Edm) - Environment Canada - Environmental Protection, Edmonton, Alberta
EC (Long)- Environment Canada - Environmental Protection, Longueuil, Quebec
EPA - U.S. Environmental Protection Agency, Corvallis, Oregon
AEC - Alberta Environmental Center, Vegreville, Alberta
ERCB - Energy Resources Conservation Board, Calgary, Alberta
ESSO - Esso Research Laboratory, Calgary, Alberta

Reference toxicants (one organic and one inorganic) were to be selected for pre-testing the toxicity procedures. Stock solutions of the reference toxicants would be centrally prepared and distributed to participating laboratories. All laboratories would be required to analyze the reference compounds and results would be reviewed prior to commencement of the sludge and centrifugate sample analyses.

Raw data were to be analyzed by all toxicity testing laboratories using the Trimmed Spearman-Karber method (Hamilton et al., 1977). LC_{50} and EC_{50}, along with the associated 95 percent confidence intervals, were to be reported for all tests. Detailed statistical analysis of reference toxicant results was not performed.

Assessment and Review of Results

A final report was to be prepared which addressed the following:

- results of all organics analyses and toxicity tests;
- summary of suitability and effectiveness of all project tasks including sampling, sample preparation, organics analyses and toxicity tests;
- interpretation of toxicity data using accepted criteria; and
- a discussion of the accuracy and cost-effectiveness of the analytical methods employed for both the organics analyses and toxicity tests.

Project Management

The project manager was to provide close communication and guidance to the participating laboratories to ensure the application of consistent testing procedures for each of the tests. The project manager was also to coordinate the following:

- site sampling;
- subsample generation;
- sample distribution;
- test timing;

- reference toxicant preparation and distribution;
- evaluation of reference toxicant results and fine-tuning of test protocols;
- compilation and evaluation of results;
- preparation of report; and
- participation at all necessary meetings and preparation of regular progress reports to the Technical Steering Committee.

1.4 PROJECT CHRONOLOGY - PHASE IIB

A summary of the major activities during the Phase IIB study is presented as follows:

1987 Oct. - Meetings to identify participating facilities, reference toxicants and to standardize test methods.

Nov. - Reconnaissance trips to three sour gas facilities, receive EPA's first set of toxicant reference results and prepare sampling procedure.

Dec. - Completed sludge sampling at the three gas plants and prepare stock solution of reference toxicant.

1988 Jan. - Receive most laboratory reference toxicant results for aquatic tests and complete centrifugation of sludges.

Feb. - Receive remaining reference toxicant aquatic test results, laboratories repeated some reference toxicant tests as a result of poor inter-laboratory agreement, and distribute sludge and centrifugate samples to laboratories.

Mar. - Eliminate one laboratory as a participant for one specific aquatic test as a result of reference toxicant testing results and commenced reference toxicant testing on terrestrial tests.

Apr. - Problems with terrestrial test organic reference toxicant concentration, begin actual sample testing on aquatic toxicity tests and receive first set of results.

May - Testing delays due to culture and transportation problems, receive further aquatic test results.

July - Organic analytical results reported by ENVIRO-TEST and additional terrestrial results.

Sept. - Review method of statistical analysis of results.

Oct. - Complete statistical analysis of toxicity test results.

Dec. - First draft of report distributed to Technical Steering Committee.

1990 May - Third and final draft of project report submitted to Technical Steering Committee and CPA Environmental Research Advisory Council for final review.

2. SLUDGE SAMPLING

Sludge sampling was conducted at three sour gas processing plants. These facilities were selected by the Technical Steering Committee to represent plants which use the three most common sweetening agents in the Canadian natural gas processing industry, namely monoethanolamine (MEA), diethanolamine (DEA) and sulfinol (the common name for sulpholane).

The candidate plants were selected in October, 1987. However, one facility initially selected from a list of volunteer CPA facilities, declined from the study. This necessitated selection of an additional facility thereby delaying commencement of the sampling program until the second week of December, 1987.

Initial contact was made with designated company representatives at each facility. A preliminary reconnaissance of the plant sites was conducted prior to the sample collection visits. During the reconnaissance trips, the specific waste sludge ponds to be sampled in the study were selected in consultation with company representatives. In addition, during the initial plant site visits there was discussion of the proposed sampling procedures, safety training requirements and specific health and safety requirements.

Preliminary sludge sample collections were obtained at two plants during the reconnaissance trips. The objectives of this sampling exercise were to:

- test proposed sampling methods;
- determine the depth of the ponds to be sampled;
- determine sludge depth in ponds; and
- obtain samples to be used to fine-tune the laboratory processing steps.

One of the major unknowns at this point in the study was the quantity of raw sludge required to meet the sludge and liquid centrifugate requirements of the analytical and toxicity testing programs. Consequently, preliminary sludge samples were centrifuged and estimates of "centrifugate" recovery made. This information was used to estimate quantities of sludge required.

Following completion of the plant reconnaissance trips, a proposed sampling procedure was developed. Copies of the procedure, which outlined the plant type, pond selected for sampling, sampling procedure, and a health and safety protection plan were submitted to the Technical Steering Committee and company representatives for comment. A copy of the proposed sampling procedure is included in Appendix A.

Sampling commenced on December 7 and was completed on December 11, 1987. Weather conditions were ideal during the sampling program. Temperatures were near the freezing point providing optimal working conditions and alleviating the need to make special arrangements to refrigerate samples.

The following sections outline aspects of the sampling program in detail.

2.1 SAMPLE REQUIREMENT

The total sample requirement was determined in consultation with the analytical laboratories following selection of the specific methods to be employed in this study. Table 2.1 outlines the list of laboratories, organic/toxicity testing procedures and individual sample requirements. The following assumptions, based on preliminary sludge sample results, were made for determining the raw sludge sample requirement:

- 1 litre of raw sludge weighed 1 kg and would generate 0.5 kg of dry material.

- 15 percent by volume of centrifugate could be recovered from each raw sludge sample.

Based on these assumptions, a total sludge requirement of approximately 100 litres, allowing approximately 10 percent for error, was estimated for the Phase IIB investigation. An additional 20 litres

Table 2.1. Sludge and liquid centrifugate requirements.

LABORATORY/ANALYSIS	SAMPLE VOLUME AND/OR MASS REQUIREMENT
ORGANICS SCREENING	
ENVIRO-TEST - Organic Screening	4 litres - homogenized sludge 1 litre - centrifugate
TOXICITY TESTING	
BIOQUEST - Daphnia	2.5 litres of centrifugate
- Earthworm	2 kilograms (dry weight) of sludge
BETA RESEARCH - Microtox	0.15 litres of centrifugate
- Seed Germination	3 kilograms (wet weight) of sludge
AEC - Seed Germination	3 kilograms (wet weight) of sludge
EPA - Algae, SOS Chromotest, Toxi-Chromotest, Daphnia	1.1 litres of centrifugate
- Earthworm	2 kilograms (dry weight) of sludge
- Seed Germination	3 kilograms (wet weight) of sludge
EC (Edm and Long) - Algae - Daphnia - Microtox - SOS Chromotest	4.5 litres of centrifugate
ERCB - Microtox	0.15 litres of centrifugate
ESSO Research - Microtox	0.15 litres of centrifugate
INORGANIC ANALYSES	
ALBERTA RESEARCH COUNCIL	5 kilograms (dry weight) of sludge

Total Centrifugate Required = 9.55 litres

Total Sludge (wet volume) = 4 litres
Total Sludge (wet weight) = 9 kilograms
Total Sludge (dry weight) = 9 kilograms

*Total Sludge Requirement = 95 litres

*Total Sludge requirement was calculated using the following assumptions.
- Raw sludge contains 15 percent centrifugate.
- 1 litre of raw sludge weighs 1 kilogram.
- Raw sludge contains 50 percent total moisture; therefore, 2 litres of raw sludge will yield 1 kilogram dry matter.

of raw sludge was collected for the Phase IIA investigation, bringing the total sample requirement of each sludge type sampled to 120 litres.

2.2 SAMPLE LOCATIONS

The following sections summarize the description of sampling locations and sludge characteristics at each plant.

2.2.1 Plant A

This location consisted of a large diethanolamine (DEA) sweetening process sour gas plant in operation since 1972. The "dry disposal pit" was selected for sampling at this location. This pit measured approximately 40 m by 25 m. It is a shallow pit, with a maximum depth of approximately 2 m . The sludge depth varied from less than 1 m to 2 m in depth.

The dry disposal pit was used as a general disposal pit over the life of the plant. It received some liquid effluent from the plant, spent catalyst, field operations liquid waste and a variety of other liquid and solid waste. The pit is connected to the plant via a small (approximately 10 cm) diameter pipeline. There was no discharge line servicing the pond.

The pond appeared relatively "dry" at the time of sampling with little surface water present. The sludge appeared as a dark, fine-textured heterogeneous material with a strong hydrocarbon odour. This sludge yielded approximately 15 percent interstitial fluid (centrifugate) on centrifugation.

2.2.2 Plant B

This location consisted of a sulfinol (the common name for sulpholane) sweetening process sour gas plant. The "burn pit" was selected for sampling. This pit was approximately 20 m square and received essentially all untreated liquid effluents from the plant. In addition, liquid hydrocarbons from the flare knock-out and process water, tank bottoms, and other waste effluents from field operations were disposed of in the pit. Hydrocarbon liquids were skimmed from the pit and the water phase was directed to either the surface water evaporation pond or to the disposal well on an as-required basis.

The plant was operating since 1968, and no cross-section drawings of the pond were available. The total depth of the pond, determined during sampling, was approximately 4.5 metres. Sludge depth varied depending on location, and the maximum depth was estimated to be approximately 3.2 metres.

The sludge material was a homogeneous black semi-solid having a strong hydrocarbon odour. This sludge yielded approximately 16 percent interstitial fluid on centrifugation.

2.2.3 Plant C

This facility was a monoethanolamine (MEA) sweetening process sour gas plant in operation since 1960. A large, approximately 40 m by 70 m, surface water holding pond was selected for sampling. This pond received overflow directly from the "flare pit" and would have received most forms of liquid effluent generated at the facility, or at field operations, over the plant's history.

The pond was approximately 3 metres deep. During sampling, the water depth in the pond was approximately 2 metres and the sludge depth varied slightly with location but averaged approximately 0.3 metres.

This location was chosen subsequent to the first two sites and therefore was not included in the initial plant reconnaissance trips, and no information was available on sludge depth or probable composition prior to sampling. The sludge was a relatively homogeneous, fine-textured, dark gray-green material with a strong hydrocarbon odour.

2.3 SAMPLE COLLECTION

2.3.1 Health and Safety

The proposed sampling procedures were reviewed with the CPA Technical Steering Committee and designated gas plant representatives prior to commencement of the sampling program. The sampling crew was required to attend a plant safety orientation meeting at each facility. The same sampling crew was employed throughout the sampling program.

Prior to sampling, personal protective measures and safety requirements were reviewed with the sampling crew and crane operators. Personal protective measures consisted of the following:

- Saranex coated Tyvek coveralls;
- disposable latex undergloves;
- nitrile gloves;
- steel-toed rubber boots;
- safety glasses and splash shields;
- hard hats; and
- half-face respirators equipped with particulate filters and organic vapour/acid gas cartridges.

This equipment was worn at all times during the sampling program.

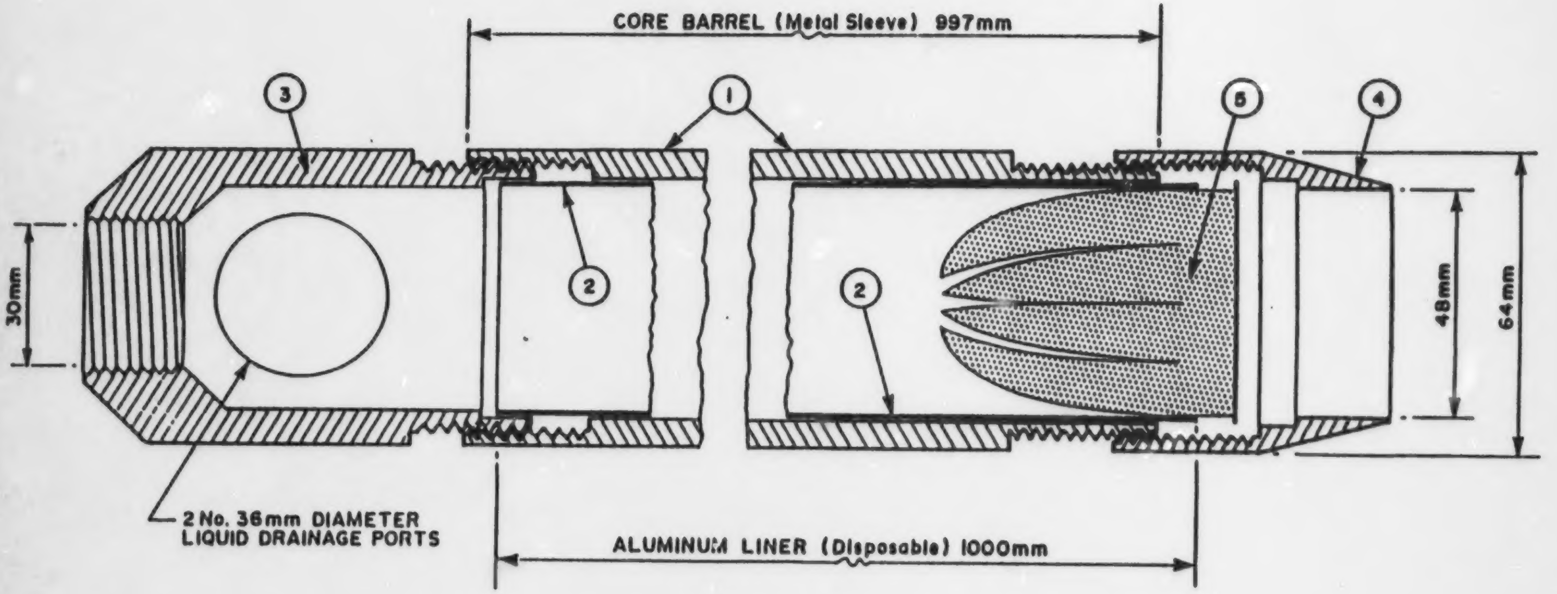
All sampling was conducted in compliance with plant safety requirements and Alberta Occupational Health and Safety Regulations.

2.3.2 Sampling Procedures

Sampling procedures varied somewhat between plants as modifications were made in the field to meet individual site requirements. The proposed sampling procedures are outlined in Appendix A.

All samples were obtained using a core sampler or dredge sampler operated from a man-basket suspended over the pond by a mobile crane. A metal core sampler was used to collect all Phase IIB samples. All Phase IIA samples were collected using a polyethylene core sampler.

The CPA core sampler (see Figure 2.1) used during the Phase II study was obtained and modified for use in this study. Because of the anticipated depth of sludges and ponds, the core barrel was


CORE BARREL (Metal Sleeve) 997mm
③
①
⑤
④
30mm
②
②
48mm
64mm
2 No. 36mm DIAMETER LIQUID DRAINAGE PORTS
ALUMINUM LINER (Disposable) 1000mm

① CORE BARREL (METAL SLEEVE)
② ALUMINUM LINER (DISPOSABLE)
③ METAL CAP
④ NOSE CONE (STAINLESS STEEL)
⑤ CORE CATCHER (PLASTIC)

FIGURE 2.1
SLUDGE CORING SYSTEM

extended to two metres. In addition, one-metre and two-metre handles were fabricated to provide flexibility in sampling. The core catcher was not available for use on this unit. The sampler was fitted with a removable 48 mm diameter aluminum sample liner.

Each pond was divided into segments and an equal number of samples were obtained from each segment to make up the total composite sample requirement. Sample recovery varied greatly from location to location depending on the depth of sludge, depth of water and sludge consistency.

The following sections summarize sampling methods at each location.

Plant A

Use of the CPA core sampler proved ineffective at this location. It was heavy to operate and the sample would not stay in the core barrel. A core catcher may have improved sample recovery. It is doubtful, however, that it would have been much more effective due to the fluid nature of the sludge.

Sampling was conducted using the 48 mm diameter aluminum liners. These cores were pressed into the sludge to depth and the top was capped with a large rubber stopper. A sample recovery bucket was suspended from the sampling platform. The core was quickly extracted from the sludge and immediately placed over the sample recovery bucket. Sample recovery was estimated at 60 to 90 percent at this location.

The pond was divided into a 6 by 10 grid with grid points spaced at 2 m and 4 m intervals along the width and length of the pond, respectively. A minimum of three samples were taken in close proximity to each grid point to recover the 100-litre sludge requirement for the Phase IIB sample requirement.

The sampling procedure was repeated at 10 grid points located along the centre of the pond, using a 48 mm diameter polyethylene core sampler to obtain samples for the Phase IIA study component.

Plant B

The pond at plant B was much deeper than anticipated from the initial site reconnaissance. The sludge was relatively homogeneous throughout its depth. Again, the CPA sludge sampler proved ineffective and sampling proceeded using the same basic procedures as employed at Plant A.

Two men were required in the man basket for sampling. The deep pond posed some unique sampling problems. For example, the rubber stopper could not be easily removed from the top of the five-metre core sampler to recover the sample. Also, when the top seal was broken, the sampler contents rushed out causing considerable splashing and some loss of sample.

The pond was divided into an eight by eight grid with a two-metre spacing between grid points. Again, a minimum of three samples was obtained from each grid point to obtain the required sample volume for Phase IIB. Ten grid points were sampled in a similar fashion using the polyethylene core sampler for the Phase IIA sample requirement.

Plant C

As indicated, due to time constraints, it was not possible to preview this sludge pond location prior to the sampling program. Unique problems were posed by the large pond size, the relatively deep water level (approximately two metres) and the shallow sludge depth (approximately 0.3 m).

The pond bottom was solid and the core sampler could not be forced into the clay liner to provide a barrier against sample loss from the sampler. Consequently, none of the core samplers would work effectively.

Samples were collected using a field-modified weighted dredge. One of the clean 20-litre alloy steel sample containers was fitted with a weighted line and used as a dredge. A heavy weight (approximately 10 kg) was fixed to the dredge line approximately one metre from the bucket handle. The weight and bucket were slowly lowered into the pond and the weight was allowed to settle into the sludge. The dredge was then slowly dragged across the bottom by swinging the crane boom. The dredge was retrieved and the sludge contents were transferred to a clean sample container.

This method worked satisfactorily at this location. The mobile crane had a maximum boom extension of 12.5 metres allowing maximum projection into the pond of approximately 10.5 metres. The pond was sampled at twelve locations around its perimeter. The sludge depth and consistency were similar at all locations. Consequently, it is anticipated that composite samples obtained from this pond are representative of overall sludge composition.

2.3.3 Sample Handling in the Field

Sample containers were filled consecutively during sample collection. Five 20-litre, clean, alloy steel containers were used to collect the Phase IIB samples, and one 20-litre high density polyethylene container was used to collect the Phase IIA sample at each site. Once full, sample containers were covered and allowed to settle until sampling was complete. Free water was decanted in the field and containers were topped-up at the completion of sampling. No attempt was made to homogenize samples in the field.

Temperatures remained cool (near freezing) throughout the sampling program and refrigeration was not required for sample preservation.

2.3.4 Sample Transport and Storage

All sample containers were marked in the field by spray-painting the plant identification code directly on the sample container. Containers were securely sealed in the field and placed in the back of a pick-up truck for transport to the laboratory.

The sampling program and sample characteristics were discussed with the Compliance Information Centre to determine appropriate requirements for sample transport under the Transportation of Dangerous Goods Regulations (TDGR). All transport was conducted in compliance with the regulations.

Samples were temporarily stored in an outside locked compound prior to transport to Edmonton. Sample containers were covered with a secured polyethylene tarpaulin during storage to maintain dark storage. Ambient temperatures during this period remained at or just below the freezing point.

All samples were transported to the ALBERTA RESEARCH COUNCIL, Hydrocarbon Laboratory, in Edmonton for processing.

2.3.5 Sample Homogenization and Splitting

A sample distribution form was developed prior to sample processing. The form identified each laboratory, analysis and sample requirement to facilitate packaging and labeling of samples. A unique sample identification number was given to each sample. Where appropriate, samples were prepared in the quantities required for a single replicate test and numbered in such a way that laboratories would not know the sample origin.

A 20-litre raw sludge sample from each plant was delivered to the ALBERTA RESEARCH COUNCIL for their Phase IIA inorganic analyses development research project and did not undergo further sample processing steps. The large quantities of raw sludge collected for this study (100 litres for each site) posed unique problems with respect to sludge sample homogenization and splitting. The combined sludge could not be effectively homogenized at one time. Therefore, the following method for sludge sample splitting was employed:

- Free water from all five 20-litre sample containers for each sludge type was decanted into 4-litre clean amber glass containers and labelled. Decanted water (ranging from 11 to 15 litres per sludge) was stored for potential future use.

- Sludge was thoroughly mixed in the five 20-litre containers using a hand-operated electric drill equipped with a stainless steel mixer.

- Measured aliquots (approximately 250 mL) from the top half of each pail were transferred to two empty pails until they were full. These pails were considered representative of the top half of the original homogenized sludge sample.

- The remaining sludge was homogenized and contents were transferred to another pail. This pail was considered representative of the remaining sludge material.

- Raw sludge samples were then prepared by taking equal aliquots from the first and second representative sludge. Originating sludges were homogenized frequently during subsampling to ensure homogeneity.

Sample containers were sealed with an aluminum foil liner and screw cap. All samples were labelled and transported to the ENVIRONMENT CANADA laboratory in Edmonton for cold storage. The remaining sludge was recombined into three 20-litre containers and stored prior to centrifugation.

Personal protective equipment, consistent with that used during sampling, was worn by technicians during sample processing.

2.3.6 Sludge Centrifugation

Sludge samples were centrifuged by the ALBERTA RESEARCH COUNCIL. Three centrifuges, each with a raw sludge capacity of one litre, were used. The homogenized sludge samples were mixed and 250 mL aliquots were loaded into centrifuge tubes. The samples were centrifuged at approximately 4000 rpm for a period of 60 minutes. The supernatants (centrifugates) were combined in four-litre, pre-cleaned amber bottles with Teflon screw caps.

The process was repeated for each sludge until a total of 12 litres of centrifugate was generated. The centrifugate was sealed, labelled and kept in cold storage awaiting sample splitting.

2.3.7 Centrifugate Homogenization and Sub-Sampling

Three 4-litre bottles of centrifugate were generated for each sludge sample site. The bottles were individually removed from the 4°C cold storage at the ALBERTA RESEARCH COUNCIL Laboratory in Edmonton, homogenized and sub-sampled in the necessary amounts required by the different Phase IIB aquatic toxicity tests.

The contents of each bottle were poured into a clean 4-litre round-bottomed distillation flask. A mixer with a stainless steel paddle was used to mix the contents of the flask for approximately five minutes. Once the sample was homogenized, sub-samples were siphoned from the flask into individual sample containers using Tygon tubing. The sub-sample sizes were 50 mL (for Microtox), 400 mL (for algae) and 800 mL (for Daphnia); the numbers of sub-samples of each size were 9, 3, and 3, respectively.

Each sub-sample of 50 mL was placed in a clean, 60-mL clear glass vial. Each of the 400-mL and 800-mL sub-samples were placed in clean 475-mL and one-litre Nalgene plastic bottles, respectively. All sub-samples were then sealed with polypropylene-lined lids and the lids secured with tape.

Before processing each sample, the siphoning apparatus was cleaned by washing with soap and tap water and then rinsing three times with tap water.

Once the initial sub-samples were prepared, they were returned to 4°C cold storage until the balance of the sub-samples were ready for packaging and transportation.

2.3.8 Sample Packaging and Distribution

All sub-samples destined for laboratories located in Alberta were placed in cardboard boxes, labelled for each laboratory, and delivered by WESTERN RESEARCH personnel.

The sub-samples were distributed in February and ambient temperatures were between 0°C and 5°C. Each individual laboratory was requested to place the sub-samples in 4°C cold storage in the dark to await testing.

Sub-samples destined for laboratories outside Alberta (i.e., BIOQUEST and U.S. EPA) were placed in 20-litre steel pails, thereby being kept in the dark. The free space in the pail was filled with absorbent material to contain any leak, and the pail was labelled in compliance with the Transportation of Dangerous Goods Regulations (TDGR).

The sub-samples sent to BIOQUEST in Winnipeg, Manitoba, were shipped via a trucking company. These sub-samples were in transit for approximately one week and were inadvertently not kept refrigerated.

Sub-samples sent to the U.S. EPA were transported by truck to Portland, Oregon. This shipment of sub-samples was held at U.S. Customs in Portland for a considerable time (three to four weeks) awaiting clearance. During the transportation and customs clearing phase, the sub-samples were not refrigerated.

After clearance through U.S. Customs, the sub-samples were transported to Corvallis, Oregon, via ground transportation.

2.3.9 Reference Toxicant Preparation and Distribution

The reference toxicant standards (reagent grade zinc chloride and 2-chloro-acetamide) were selected and used in a preliminary round of testing which involved all participating toxicity testing laboratories. The purpose of the reference toxicant testing round was to ensure that all laboratories could conduct the toxicity test procedures without any problems, prior to receiving actual gas plant sludge and centrifugate samples for testing. Reference toxicant stock solutions for participating Canadian laboratories were prepared by WESTERN RESEARCH following instructions obtained from the U.S. EPA Corvallis Laboratory. The U.S. EPA Corvallis laboratory prepared their own stock solutions. The zinc chloride stock solution was prepared to a concentration of 3,700 mg/L, as zinc. The 2-chloroacetamide stock solution was prepared from stock crystals supplied by the U.S. EPA to a concentration of 5,000 mg/L.

Reference toxicant solutions were mixed thoroughly and subdivided for use by individual laboratories. All solutions were kept refrigerated prior to distribution. The reference solutions were hand-delivered to the local laboratories and packaged and labelled according to TDG regulations for shipment to out-of-town laboratories. Air freight embargos due to the Christmas season caused a change in plans. Consequently, samples were shipped to laboratories by ground courier and, as a result, considerable delay was experienced (up to four weeks).

2.3.10 Pre-Testing Treatment of Multiphasic Centrifugate Samples

The following pre-test procedure was agreed to by all participating aquatic toxicity testing laboratories for centrifugate samples which are multiphasic, i.e. separate into oil and water phases:

- Sample bottles are to be agitated for one minute then transfer contents into a separatory funnel and allow to sit for 24 hours in a 4°C refrigerator.
- After 24 hours, draw off the aqueous phase, leaving the oil phase. Retain oil phase for possible future analysis.

This procedure was followed for the Site A centrifugate samples.

3. ORGANIC ANALYSES

The objective of this component of the study was to develop a more effective analytical protocol for the organic analysis of complex gas plant sludge samples than the approach used in the original Phase II study. Critical aspects of this protocol were reproducibility of results and cost effectiveness.

The organic analysis study evaluated whole sludge and centrifugate samples. Major aspects of this portion of the study were:

- development of an analytical method to determine the presence of volatile and semi-volatile priority pollutants in gas plant sludge and centrifugate samples;
- development of an analytical method to determine diethylene glycol (DEG), monoethanolamine (MEA), diethanolamine (DEA) and sulfinol (SUL) in appropriate samples of gas plant sludge and centrifugate;
- to demonstrate a more cost-effective approach to chemically identifying toxic organic contaminants in gas plant sludge and centrifugate samples;
- testing the validity of these methods by submitting duplicate samples of sludge and centrifugate to a designated laboratory, and comparing the results.

Development and evaluation of the procedures was conducted primarily by ENVIRO-TEST LABORATORIES in Edmonton, with duplicate sample analysis work the responsibility of KANANASKIS CENTRE FOR ENVIRONMENTAL RESEARCH (KCER) in Calgary.

3.1 LITERATURE SURVEY

A literature search was undertaken to determine the most effective method for the identification of MEA, DEA and associated degradation products. Chemical Abstract Numbers (1967-1987) were manually searched. A total of 270 references were obtained and possible methods retrieved are described as follows:

1) Direct Aqueous Injection:

These methods involve gas chromatography/mass spectrometry detection (GC/MS) and gas chromatography/flame ionization detection (GC/FID) analysis of aqueous solutions of DEA and associated degradation products using a 183 cm by 3 mm O.D. stainless steel column packed with Tenax GC. Previous experience with this method revealed poor resolution when applied to the analysis of reclaimed amine solutions.

2) Derivatization - Gas Chromatography:

Several methods followed this approach; derivatization included silylation and trifuoroacetylation. Both these methods require water removal prior to derivatization. This is usually accomplished by heating the sample and air stripping the water, resulting in substantial losses of volatile alkanolamines such as MEA and DEA. One silylation method, however, eliminated the need to remove water but is limited to small sample sizes (maximum of 25 μL), hence detection limits are high. This method is useful for unambiguous identification by GC/MS of alkanolamines in samples containing high levels, i.e., greater than 500 ppm.

3) Derivatization - High Performance Liquid Chromatography (HPLC):

Two methods were revealed that appeared promising and they are:

i) Derivatization of the alcohol functional group of alkanolamine with 3,5 - dinitrobenzoyl chloride and analysis by HPLC-UV detection (Valdez and Reier, 1986). This method was attempted in the lab but would not successfully operate, likely due to decomposed reagent.

ii) Derivatization of primary and secondary amines with 9-fluorenylmethyl chloroformate (FMOC) and analysis using HPLC-fluorescence detection (Nasholm et al, 1987 and Einarsson et al, 1986). This method was tested using MEA, DEA, N,N'-bis (2-hydroxyethyl) ethylene diamine (BHEED) and THEED and it was found to be sensitive, accurate and precise.

The HPLC method exhibited detection limits of 0.2 mg/L for liquids and 0.2 µg/g for solids, based on 10 mL or 10 g sample sizes, respectively. Because of its sensitivity, the second HPLC-derivatization method was selected for the analysis of sludges and centrifugates for MEA, DEA and degradation products.

Analysis for DEG utilized a routine GC/MS scan of the methanol extract while sulfinol was evaluated using standard GC/FPD analysis of the same extract.

3.2 SUMMARY OF ORGANIC CONTAMINANT ANALYTICAL PROGRAM

Sludge and centrifugate samples from each of three gas plant waste locations were subjected to various methods of extraction and analyses to identify volatile and semi-volatile priority pollutants and water soluble (intractable) components. Analyses were also performed to characterize the conventional parameters. Appropriate calibration components were included in the analyses to permit quantification of identified compounds and validation of the method.

3.2.1 Sample Preparation, Extraction and Analyses - Sludges

Samples were collected, homogenized and subsampled as discussed in Section 2. All attempts were made to maintain samples at 4°C when in storage and to expedite transport.

3.2.1.1 Sludge analysis for semi-volatile priority pollutants. The method of generation of the various fractions for analysis of the sludge for semi-volatile priority pollutants is presented in Figure 3.1. An appropriate amount of surrogate cocktail (Table 3.1) was added prior to the overnight Soxhlet extraction with dichloromethane (DCM).

The alumina adsorbent used for the column generation of most of the fractions required preparation and calibration prior to the actual sample application. Preparation of the alumina (neutral, 70-230 mesh, Merck) consisted of overnight Soxhlet extraction with DCM, removal of solvent by overnight vacuum desiccation, measuring 10.0 grams quantities of dry adsorbent into scintillation vials and activation at 400°C overnight. Once the alumina was activated, the adsorbent could be calibrated. This was achieved by mixing 100 µL, 150 µL, 200 µL and 250 µL of organic-free water with four 10.0 gram aliquots of activated adsorbent, transferring 6 grams of this to four 10-mm I.D. chromatography columns, adding 250 µL of calibration mixture (Table 3.2) to 3 g of the remaining adsorbent, mixing this and adding it to the appropriately deactivated alumina column. The columns were then successively eluted with 20 mL pentane, 50 mL benzene, 100 mL chloroform and 200 mL methanol containing 5% water (v/v).

The fractions were concentrated to 1.0 mL (2.0 mL for sample fractions) using rotary evaporation and nitrogen blowdown. The pentane was exchanged into hexane prior to analysis by adding excess hexane and concentrating to 1.0 mL. The methanol/5% water fraction was concentrated to 2.0 mL with rotary evaporation, diluted to 4.0 mL with organic-free water and acetylated. Acetylation of this fraction was achieved by adding 250 µL concentrated acetic anhydride to the 4.0 mL of the fraction (water as solvent), adding solid sodium bicarbonate slowly, and mixing until evolution of carbon dioxide ceases. This was then extracted three times with 2 mL of chloroform, dried by passage through a 1-gram sodium sulfate column and

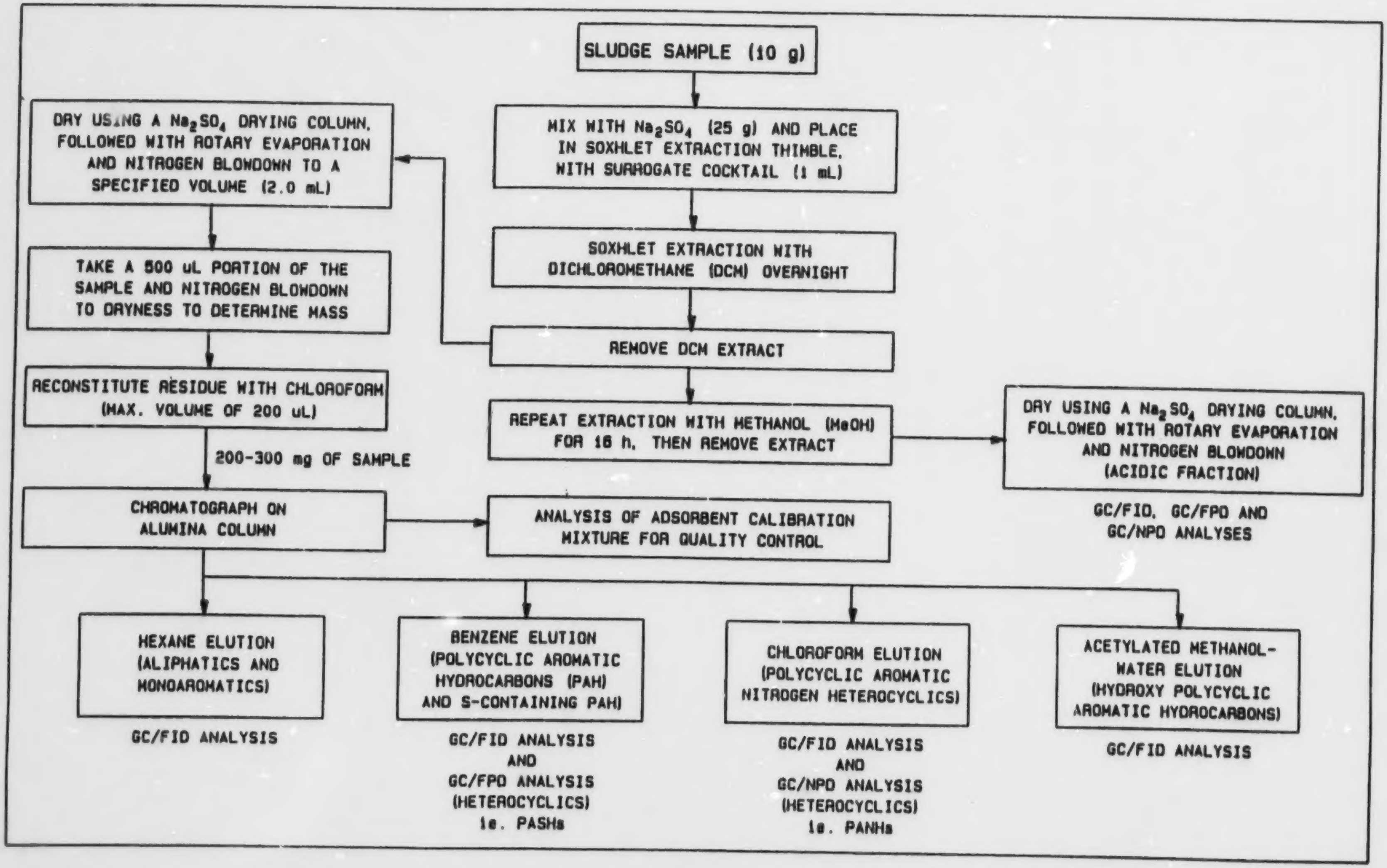

SLUDGE SAMPLE (10 g)
MIX WITH Na_2SO_4 (25 g) AND PLACE IN SOXHLET EXTRACTION THIMBLE, WITH SURROGATE COCKTAIL (1 mL)
SOXHLET EXTRACTION WITH DICHLOROMETHANE (DCM) OVERNIGHT
REMOVE DCM EXTRACT
REPEAT EXTRACTION WITH METHANOL (MeOH) FOR 16 h, THEN REMOVE EXTRACT
DRY USING A Na_2SO_4 DRYING COLUMN, FOLLOWED WITH ROTARY EVAPORATION AND NITROGEN BLOWDOWN (ACIDIC FRACTION)
GC/FID, GC/FPD AND GC/NPD ANALYSES
DRY USING A Na_2SO_4 DRYING COLUMN, FOLLOWED WITH ROTARY EVAPORATION AND NITROGEN BLOWDOWN TO A SPECIFIED VOLUME (2.0 mL)
TAKE A 500 uL PORTION OF THE SAMPLE AND NITROGEN BLOWDOWN TO DRYNESS TO DETERMINE MASS
RECONSTITUTE RESIDUE WITH CHLOROFORM (MAX. VOLUME OF 200 uL)
200-300 mg OF SAMPLE
CHROMATOGRAPH ON ALUMINA COLUMN
ANALYSIS OF ADSORBENT CALIBRATION MIXTURE FOR QUALITY CONTROL
HEXANE ELUTION (ALIPHATICS AND MONOAROMATICS)
GC/FID ANALYSIS
BENZENE ELUTION (POLYCYCLIC AROMATIC HYDROCARBONS (PAH) AND S-CONTAINING PAH)
GC/FID ANALYSIS AND GC/FPD ANALYSIS (HETEROCYCLICS) ie. PASHs
CHLOROFORM ELUTION (POLYCYCLIC AROMATIC NITROGEN HETEROCYCLICS)
GC/FID ANALYSIS AND GC/NPD ANALYSIS (HETEROCYCLICS) ie. PANHs
ACETYLATED METHANOL-WATER ELUTION (HYDROXY POLYCYCLIC AROMATIC HYDROCARBONS)
GC/FID ANALYSIS

FIGURE 3.1 ANALYSIS OF GAS PLANT SLUDGE FOR SEMI-VOLATILE PRIORITY POLLUTANTS

Table 3.1. Constituents of surrogate mix.

Analyte	Concentration (µg/mL)
d_8-Naphthalene	1011
d_7-Quinoline	1011
4-Bromophenol	1010
4-Fluorobiphenyl	1003
d_{10}-Biphenyl	980
d_{10}-Acenaphthene	1014
d_8-Dibenzothiophene	806
d_{12}-Chrysene	753

Table 3.2. Constituents of adsorbent column calibration mixture.

Analyte	Concentration (µg/mL)	Expected Fraction	Expected Recovery (%)
n-Pentacosane	456	pentane	80-100
7,8-Benzoquinoline	453	chloroform	80-100
3-Aminofluoranthene	440	chloroform	80-100
2-Naphthol	463	methanol/ water	80-100
2-Methylindole	457	chloroform	80-100
Benzo (g,h,i) perylene	472	benzene	80-100
Phenanthrene	548	benzene	80-100

concentrated to 1.0 mL using nitrogen. An acetylated phenol calibration standard was prepared by adding 25 µL of concentrated 2-naphthol and 10 µL of concentrated 4-bromophenol to 4 mL of organic free water, mixing and acetylating, as for the methanol/water fraction.

All fractions, as well as the calibration mixture and the acetylated phenol calibration standard, were analyzed by gas chromatography/flame ionization detection (GC/FID), noting the recoveries of analytes and the fractions in which they occur

Analysis of the data revealed that deactivation with 200 µL of water resulted in satisfactory separation and recovery of the model compounds present in the calibration mixture. Criteria for this separation and expected recoveries are presented in Table 3.2. As a result of this validation, activated alumina was deactivated with 200 µL of water prior to chromatography of sample abstracts.

Once the alumina column adsorbent was calibrated, the samples were fractionated and analyzed. A 500 µL aliquot of the Soxhlet dichloromethane extract was blown dry with nitrogen and the weight determined. This sample was reconstituted with 200 µL of chloroform and applied to the 3 grams of prepared alumina (2% water deactivated, i.e. 200 µL water added to 10 g) in a scintillation vial. To ensure transfer of the residue to the adsorbent, the sample container was then rinsed twice with 500 µL of pentane (the rinsings applied to the adsorbent). The solvent was removed from the adsorbent using a gentle stream of nitrogen and rotating the vial until a free flowing mixture was obtained. This adsorbent was then applied to the alumina column and eluted, as discussed for the column calibration, to generate the fractions for analysis for semi-volatiles.

GC/FID analysis was performed on all fractions to determine proper elution of each prior to GC/MS analysis and to determine if enough material is present in the fraction to warrant GC/MS. Qualitative analyses, i.e. identification of the major components, was conducted by retrieving scanning data and comparing the observed mass spectra to those found in reference libraries, such as the NBS library, Eight Peak Index of Mass Spectra, Wiley's Library of Mass Spectra, and by manual interpretation from first principles.

3.2.1.2 Sludge analysis for volatile priority pollutants. Preparation of the sludges for the determination of benzene, toluene, ethylbenzene and xylenes (BTEX) consisted of measuring known sample amounts into vials, adding an internal standard, heating, and analyzing a headspace sample.

Specifically, sample was added to 25 mL of organic-free water until the water level reached a previously determined level equivalent to 35 mL, and weighed to establish sample weight. Standards were prepared by adding sand in place of sample and injecting known amounts of benzene, toluene, ethylbenzene, and xylene. An internal standard (chlorobenzene) was injected into all sample and standard tubes. The tubes were then heated at 55-60°C for 10 minutes, shaken and heated a further 30 minutes. Headspace samples were removed and analyzed with GC/FID chromatography.

3.2.1.3 Sludge analysis for water soluble compounds. Analysis for each of the water soluble compounds, monoethanolamine (MEA), diethanolamine (DEA), sulfinol and diethylene glycol (DEG) followed a method summarized in Figure 3.2 and the method for MEA and DEA analysis was derived from the methods of Nasholm et al (1987) and Einarsson et al (1986).

Initial extraction of the sludge sample for water soluble components was accomplished by mixing 10 grams of wet sludge with 6 grams celite (previously purified with methanol extraction), packing into an all glass Soxhlet extraction thimble, extracting with methanol overnight, concentrating the methanol to 5 mL and adjusting the final volume to 10 mL with methanol.

The methanol extract was analyzed for sulfinol using gas chromatography/flame photometric detection (GC/FPD).

After addition of nitrobenzene as an internal standard, the methanol extract was also analyzed for diethylene glycol using GC/MS.

Analysis for MEA and DEA followed the method of Einarsson et al (1986) and Nasholm et al (1987). Sample extracts and standard solutions of MEA and DEA (in methanol) were analyzed. Briefly,

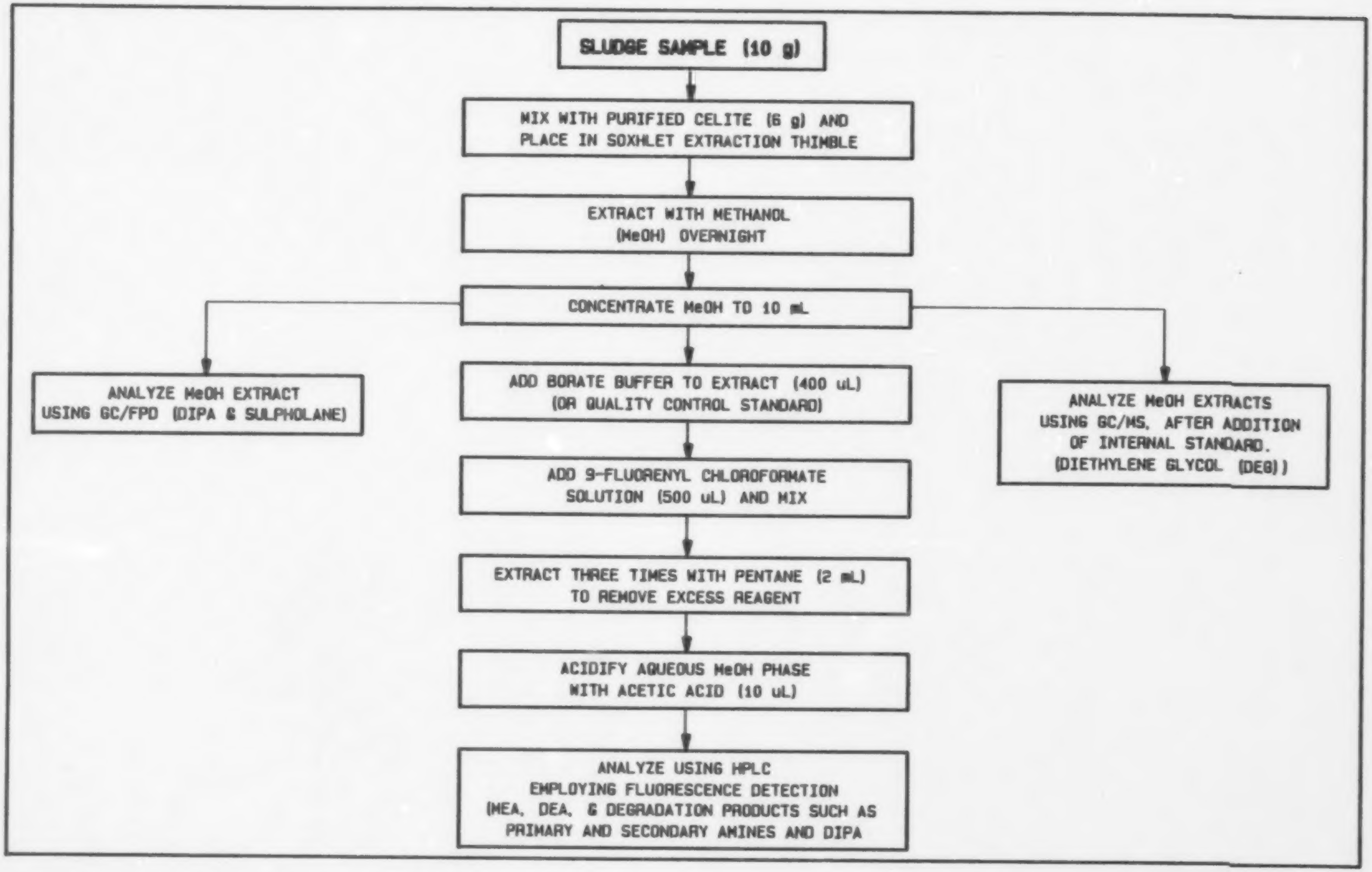

SLUDGE SAMPLE (10 g)
MIX WITH PURIFIED CELITE (6 g) AND PLACE IN SOXHLET EXTRACTION THIMBLE
EXTRACT WITH METHANOL (MeOH) OVERNIGHT
CONCENTRATE MeOH TO 10 mL
ANALYZE MeOH EXTRACT USING GC/FPD (DIPA & SULPHOLANE)
ADD BORATE BUFFER TO EXTRACT (400 uL) (OR QUALITY CONTROL STANDARD)
ANALYZE MeOH EXTRACTS USING GC/MS, AFTER ADDITION OF INTERNAL STANDARD. (DIETHYLENE GLYCOL (DEG))
ADD 9-FLUORENYL CHLOROFORMATE SOLUTION (500 uL) AND MIX
EXTRACT THREE TIMES WITH PENTANE (2 mL) TO REMOVE EXCESS REAGENT
ACIDIFY AQUEOUS MeOH PHASE WITH ACETIC ACID (10 uL)
ANALYZE USING HPLC EMPLOYING FLUORESCENCE DETECTION (MEA, DEA, & DEGRADATION PRODUCTS SUCH AS PRIMARY AND SECONDARY AMINES AND DIPA

FIGURE 3.2 ANALYSIS OF GAS PLANT SLUDGE FOR WATER SOLUBLE COMPOUNDS

100 µL of borate buffer (pH 7.9, 1M) was added to 400 µL of sample (or standard) and mixed; 500 µL of 9-fluorenyl chloroformate (15 mM in acetonitrile:acetone, 1:1) added and mixed; excess reagent was removed by three successive extractions with pentane (2 mL each), and then the aqueous methanol phase acidified by adding 10 µL of concentrated glacial acetic acid. Chromatographic analysis of the samples was performed using a high pressure liquid chromatograph employing fluorescence detection.

Quality control was ensured by duplicate analysis of one of the site samples fortified with MEA, DEA, sulfinol and DEG at two levels. All fortified samples were analyzed and the recoveries were noted.

3.2.1.4 Extraction of sludge samples for alkanolamine degradation products. Wet sludge (10.0 g) was mixed with six grams of purified Celite and packed into a glass Soxhlet extraction thimble. After extracting the sample overnight with methanol, the resulting extract was concentrated to 100.0 mL.

3.2.1.5 Quality control for alkanolamine degradation products. Sample C was fortified with the following alkanolamine degradation products and the recovery noted. Appropriate method blanks were also determined in order to check for artifacts introduced during the sample processing and workup. The alkanolamine degradation products employed include:

Chemical Name	Abbreviation
Diethanolamine	DEA
N-(hydroxyethyl)ethylenimine	HEM
Triethanolamine	TEA
Oxazolidone	OX
N-(hydroxyethyl)piperazine	HEP
N,N'-bis-(hydroxyethyl)piperazine	BHEP

Fortified sludge samples were analyzed using the method of Valdez (1985) and gas chromatography/flame ionization detection (GC/FID).

3.2.1.6 Derivatization of centrifugates and sludge extracts for analysis of alkanolamine degradation products. Two derivatization methods were employed for the analysis of alkanolamine degradation products in sludge extracts and aqueous centrifugate samples.

The first method employed was that described by Valdez (1985). Briefly, 25 µL of sludge extract (concentrated a further 25 fold) and/or aqueous centrifugate sample was added to a 3.5 mL septum cap vial and the contents mixed with 175 µL of dry acetonitrile. After the addition of 500 µL of N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA) containing one percent trimethylchlorosilane (TMCS) the mixture was capped, sealed with teflon tape, and heated in an aluminum block maintained at 60°C overnight. The resulting mixture was analyzed using gas chromatography/flame ionization detection (GC/FID). Conditions for analysis are described in Section 3.2.4.1.

Aqueous solutions of DEA, HEM, OX, BHEP, HEP, and TEA were prepared from neat material and derivization and analyzed by GC/FID as described above.

Another derivatization procedure employed was that described by Einarsson et al. (1986) and Nasholm et al. (1987). Sludge extract and/or aqueous centrifugate (400 µL) were derivatized with 9-fluorenymethyl chloroformate (FMOC) and the resulting derivative analyzed using high pressure liquid chromatography with fluorescence detection. Even though this method is very sensitive, it is only applicable to alkanolamine degradation products which are either primary or secondary amines. Alkanolamine degradation products which could be analyzed by this method include: N,N'-bis-(hydroxylethyl) ethylenediamine (BHEED), diethanolamine (DEA), N-(hydroxyethyl) ethylenediamine (HEED), N-(hydroxyethyl)piperazine (HEP), monoethanolamine (MEA), N,N,N'-tris-(hydroxyethyl)ethylenediamine (THEED) and diisopropanolamine (DIPA). This method is more fully described in Section 3.2.1.3.

3.2.2 Sample Preparation, Extraction, and Analysis - Centrifugates

Centrifugate was generated from homogenized sludge samples, as described in Section 2.3.6. Storage of the sealed samples was at 4°C until transport to the appropriate laboratories for analysis.

3.2.2.1 Centrifugate analysis for semi-volatile priority pollutants. One-litre aliquots of centrifugate or, in the case of oily centrifugates, aliquots (100 mL) of centrifugate diluted to 1 litre with organic free water, were analyzed using U.S. EPA Method 625 (Federal Register, 1984). Briefly, samples were extracted serially under basic and then acidic pH with dichloromethane. Both the base/neutral (B/N) and acid extracts were dried by passage through anhydrous sodium sulfate and concentrated to 2 mL. An aliquot (500 µL) of the B/N extract was subjected to chromatography on alumina following the procedures outlined in Section 3.2.1.1.

All fractions obtained were subjected to GC/FID analysis and from these data decisions with respect to dilution and further GC/MS analysis were made. Fractions which contained few GC peaks as revealed by GC/FID analysis were not subjected to further analysis. Fractions which contained substantial GC peaks were subjected to further GC/MS analysis. Both quantitative (i.e., U.S. EPA semi-volatile priority pollutant) and qualitative (i.e., identification of major components) information was sought using GC/MS analysis.

As an aid to qualitative analysis using GC/MS, all benzene fractions from the alumina chromatography were analyzed by gas chromatography/flame photometric detection (GC/FPD) for the presence of PASH's, and all chloroform fractions from alumina chromatography were analyzed by gas chromatography/nitrogen-phosphorous detection (GC/NPD) for the presence of PANH's.

Quality control for the centrifugate analysis was provided by fortifying the samples with the surrogate compounds presented in Table 3.2, and noting the recoveries of these compounds from the fortified samples.

3.2.2.2 Centrifugate analysis for volatile priority pollutants. Analysis was performed on 40-mL volumes of the samples and standards (organic-free water spiked with methanol solutions of known amounts of benzene, toluene, ethylbenzene and xylene). These volumes were contained in 60 mL culture tubes sealed with septum caps. The tubes were heated to 55-60°C and maintained at temperature for 20 minutes. Analysis using GC/FID was performed on 5-mL samples of the tube headspaces.

3.2.2.3 Centrifugate analysis for water soluble compounds. The method presented for sludge analysis of water soluble compounds in Section 3.2.1.3 was also utilized to analyze the centrifugate samples.

3.2.2.4 Analysis of centrifugates for alkanolamine degradation products. Aqueous centrifugate samples were used directly for the determination of alkanolamine degradation products. Derivatization of the extract is described in Section 3.2.1.6.

3.2.3 Analyses of Sludge and Centrifugates for Conventional Inorganic Parameters

Samples of sludge and centrifugate were analyzed for conductivity, nitrate/nitrite nitrogen, ammonia nitrogen, TKN nitrogen, total organic carbon (TOC), phenol and sulfide by NORWEST LABORATORIES using U.S. EPA and/or ASTM-approved methodology.

3.2.4 Chromatographic Conditions

Chromatographic analyses were conducted using apparatus and conditions as outlined in the following sections.

3.2.4.1 Gas chromatography/flame ionization detection (GC/FID) for semi-volatile priority pollutants. A 30 m by 0.25 mm I.D. fused silica capillary column coated with DB-5 (0.25 μm film thickness, J&W SCIENTIFIC) was employed for the analysis.

The GC/FID conditions were 40°C for 4 minutes, an increase of 10°C/min to 300°C and hold for 5 minutes. The injector and detector were maintained at 250° and 300°C, respectively. Two µL of sample were injected via a capillary injector operated in the splitless mode. The injector was purged with carrier gas (helium) 30 seconds after injection. The linear velocity of the carrier gas was maintained at 25 cm/sec at 300°C.

GC conditions for GC/FID, GC/FPD, GC/NPD and GC/MS analyses for semi-volatile components should be the same in order to allow for inter-comparison of the data and assist with the qualitative GC/MS analysis.

3.2.4.2 Gas chromatography/flame ionization detection (GC/FID) for volatile priority pollutants. A 183 cm by 3 mm stainless steel column packed with 1% SP 1000 coated on Carbopack B (SUPELCO, INC.) was used for the analysis. The GC oven conditions were 45°C (no initial hold time) to 220°C at 15°C/min with a final hold for 20 minutes. The injector and detector temperatures were 150° and 210°C, respectively. The carrier gas was helium and the flow was maintained at 40 mL/minute.

3.2.4.3 Gas chromatography/flame photometric detection (GC/FPD) for sulfinol. Oven temperature conditions for analysis were 50°C for 1 minute, 9°C/min to 220°C and hold for 5 minutes. Injector and detector temperatures were 250° and 220°C, respectively. The analytical column was a 25 m by 0.31 mm I.D. fused silica capillary column coated with SE-54 (HEWLETT-PACKARD). The stationary phase was cross-linked and the film thickness was 0.17 µm. The carrier gas was helium and the column pressure was adjusted to maintain a linear velocity of 25 cm/min at 220°C.

3.2.4.4 Gas chromatography/mass spectrometry detection (GC/MS) for diethylene glycol (DEG). Oven temperature conditions for analysis were 50°C for 3 minutes, 10°C/min. to 220°C and hold for 5 minutes. The column employed was a 30 m by 0.25 mm I.D. fused silica capillary column coated with Carbowax 20 M (J & W SCIENTIFIC). The carrier gas was helium and the column pressure was adjusted to maintain a linear velocity of 25 cm/sec at 220°C. Mass spectrometry analysis was conducted using selected ion monitoring techniques; ions monitored included m/z 77,123 (internal standard), and 75 and 45 (DEG). Quantification ions employed for DEG and the internal standard were m/z 45 and 77, respectively.

3.2.4.5 High pressure liquid chromatography (HPLC) detection for monoethanolamine (MEA) and diethanolamine (DEA). HPLC conditions employed the following gradient: 75% solvent A (3 mL glacial acetic acid in 1 litre of water with pH adjusted to 4.2 using 6N sodium hydroxide) and 25% solvent B (HPLC grade acetonitrile) for 2 minutes, increasing the solvent strength linearly over 18 minutes to 75% B and 25% A. Solvent was then changed to the initial conditions (75% A, 25% B) over 0.5 minutes. Fluorescence detection was at 249 nm (excitation wavelength) and emission was measured above 370 nm. The range on the detecter was 0.1.

3.2.4.6 Detection limits. The expected detection limits for the EPA target priority pollutants during the GC/MS analysis are presented in Table 3.3.

3.3 INTER-LABORATORY METHOD DIFFERENCES

KANANASKIS CENTRE FOR ENVIRONMENTAL RESEARCH (KCER) was provided with one sample each of sludge and centrifugate and conducted analyses similar to the protocol described in Section 3.2. KCER variations from this protocol are listed below:

- alumina column calibration was not performed in a manner comparable to the ENVIRO-TEST calibration.

- 200 µL, rather than 250 µL, alumina column calibration mixture was used.

Table 3.3. EPA target compound priority pollutant compounds analyzed by GC/MS at ENVIRO-TEST - EPA method 625 and method detection limits[1].

Base/Neutral Fraction	Detection Limit (µg/L)	Acid (Phenols) Fraction	Detection Limit (µg/L)
Benzidine	50	2-Chlorophenol	10
Bis(2-chloroethyoxy)methane	10	2,4-Dichlorophenol	10
Bis(2-chloroethyl)ether	10	2,4-Dimethylphenol	10
Bis(2-chloroisopropyl)ether	10	4,6-Dinitro-o-cresol	20
Bis(2-ethylhexyl)phthalate	10	2,4-Dinitro-phenol	20
4-Bromophenyl phenyl ether	10	2-Nitrophenol	20
Butyl benzyl phthalate	10	4-Nitrophenol	50
2-Chloronaphthalene	10	p-Chloro-m-cresol	10
4-Chlorophenyl phenyl ether	10	Pentachlorophenol	50
1,2-Dichlorobenzene	10	Phenol	10
1,3-Dichlorobenzene	10	2,4,6-Trichlorophenol	10
3,3'-Dichlorobenzidine	50		
Dimethylphthalate	10		
Diethylphthalate	10		
Di-n-butylphthalate	10		
2,4-Dinitrotoluene	10		
2,6-Dinitrotoluene	10		
Hexachlorobenzene	10		
Hexachlorobutadiene	10		
Hexachorocyclopentadiene	10		
Isophorone	10		
Nitrobenzene	10		
N-Nitrosodiphenylamine	20		
N-Nitrosodimethylamine	50		
N-Nitrosodi-n-propylamine	20		
1,2,4-Trichlorobenzene	10		
1,4-Dichlorobenzene	10		
Hexachloroethane	10		

Note: These detection limits apply to 1 L of liquid sample extracted with solvent and concentrated to a 1.0 mL final volume.

... continued

Table 3.3. EPA target compound priority pollutant compounds analyzed by GC/MS at ENVIRO-TEST - EPA method 625 and method detection limits (concluded).

Polycyclic Aromatics Fraction	Detection Limit (μg/L)	Pesticides Fraction	Detection Limit[2] (μg/L)
Acenaphthene	10	Aldrin	1.9
Acenaphthylene	10	BHC	4.2
Anthracene	10	4,4'-DDT	4.7
Benzo(a)anthracene or	10	4,4'-DDE	5.6
Chrysene		4,4'-DDD	2.8
Benzo(k)fluoranthene or	10	Dieldrin	2.5
Benzo(b)fluoranthene		Heptachlor	1.9
Benzo(a)pyrene	10	Heptachlor epoxide	2.2
Fluoranthene	10	PCB-1242	2.5
Fluorene	10	PCB-1254	36
Naphthalene	10	PCB-1221	30
Phenanthrene	10		
Pyrene	10		

Notes: - These detection limits are based on 1L of sample and an extract volume of 1.0 mL.
- Compounds do not include priority metals (inorganics) or purgeable organics.

[1] Detection limit obtained from Federal Register: vol. 49, pg. 184-199 (i.e. Method 1625, 1984).

[2] Detection limit obtained from Federal Register: vol. 49, pg. 153-174 (i.e. Method 625, 1984); and also Environ. Sci. and Technol. (1981) vol. 15, pg. 1426-1435.

- gas chromatograph (VARIAN 3400 for FID or HP5890 for FPD and NPD) contained a 15-m SE54 narrow-bore capillary column.

- the DCM extract from the sludge sample could not be concentrated to less than 2.4 mL. The remaining procedure was followed, as described in Section 3.2.1.1.

- GC/MS analyses were carried out using a FINNIGAN 4500 GC/MS. Gas chromatography was performed using a 30-m x 0.25 mm I.D. SE-54 capillary column.

- HPLC analysis for MEA and DEA and analysis for volatile components were not performed.

The sludge sample analyzed by KCER was sludge from Plant A. KCER also analyzed the centrifugate sample from Plant C.

3.4 RESULTS

3.4.1 Quality Control for Sludge Sample Analyses

Alumina was validated by spiking batches of alumina with the compounds presented in Table 3.2 and noting the recovery and fractions associated with various model compounds. Surrogates were added to all the sludge samples, blanks and an alumina column spike. The recovery of surrogates was noted in all samples analyzed. Water soluble compounds were added to aliquots of the Site B sample (since it had the least organics and the most water soluble compounds), in duplicate, at two levels, and the recoveries were noted. Recoveries of surrogates from sludge samples were evaluated by both ENVIRO-TEST and KCER.

3.4.1.1 Validation of alumina column. The results of the alumina column validation are presented in Table 3.4.

Table 3.4. Recoveries of calibration compounds from alumina column.

			ENVIRO-TEST	KCER
Class	Compound	Fraction	% Recovery	% Recovery
Saturate	n-Pentacosane	Pentane	100	70
		Benzene	-	1
PAH	Phenanthrene	Benzene	96	90
PAH	Benzo (g,h,i) perylene	Benzene	98	-
		Chloroform	-	98
PANH	2-Methylindole	Chloroform	22	3
		Benzene	-	24
PANH	3-Aminofluorathene	Chloroform	86	42
PANH	7,8-Benzoquinoline	Chloroform	99	-
Hydroxy-PAH	2-Naphthol	Methanol:Water	100	77

These results indicate that ENVIRO-TEST achieved a correct alumina activation level since excellent separation of saturates (e.g., n-pentacosane), polycyclic aromatic hydrocarbons (e.g., phenanthrene and benzo (g,h,i) perylene), polycyclic aromatic nitrogen heterocycles (e.g., 2 - methylindole, 3 - aminofluoranthene and 7,8-benzoquinoline) and hydroxy-polycyclic aromatic hydrocarbons (eg., 2-naphthol) was achieved. Recoveries were excellent except for 2-methylindole. The low recovery for 2-methylindole was attributed to volatilization during solvent removal from the adsorbent and it was suggested that solvent be directly applied to the adsorbent (to a maximum of 200-300 µL) and chromatographed without prior solvent removal, i.e. reconstitute the residue with 200-300 µL $CHCl_3$ and run chromatograph.

The results from KCER indicate that some compounds were recovered in the improper fractions, e.g., 2-methylindole, benzo (g,h,i) perylene and, to a minor degree, n-pentacosane. These results were obtained with a 1% deactivation of the alumina. A previous attempt by KCER with 2% deactivated alumina resulted in more significant occurrences of compounds eluting in improper fractions.

It was also noted that the KCER recoveries were low in comparison to those of ENVIRO-TEST. KCER performed a single set of analyses and the low recoveries may be the result of procedural problems.

3.4.1.2 Recovery of surrogates from sludge. The ENVIRO-TEST recoveries of the deuterated standards (Table 3.1) from the fractionation of the sludge, alumina column spikes and method blanks and KCER's recovery of standards from fractionation of the sludge DCM extract are presented in Table 3.5.

All recoveries for the ENVIRO-TEST analyses were well above the desired recovery of greater than 80% for the deuterated internal standards. It was also observed that surrogates eluted in alumina fractions consistently with their chemical class; that is, naphthalene-d_8, 4-fluorobiphenyl, dibenzothiophene-d_8, biphenyl-d_{10},

Table 3.5 Surrogate recovery from sludges.

Standard	ENVIRO-TEST % Recovery (mean)	 RSD[1] (%)	KCER % Recovery
Naphthalene - d_8	97	15	14,26[2]
4 - Bromophenol	96	24	30
4 - Fluorobiphenyl	117	30	54
Dibenzothiophene - d_8	122	19	139
Biphenyl - d_{10}	121	26	85
Quinoline - d_7	85	25	81
Chrysene - d_{12}	104	9	123
Acenaphthalene - d_{10}	126	26	86

[1] Relative Standard Deviation, n=5.

[2] These represent the recoveries for the pentane and benzene fractions, respectively.

chrysene-d_{12} and acenaphthalene-d_{10} consistently appeared in the benzene fraction (polycyclic aromatic hydrocarbons and polycyclic aromatic sulfur heterocycles). Quinoline-d_7 appeared in the chloroform fraction (polycyclic aromatic nitrogen heterocycles) and 4-bromophenol appeared in its acetylated form in the methanol-water fraction (hydroxy polycyclic aromatic hydrocarbons). The recoveries presented in Table 3.4 indicate that ENVIRO-TEST achieved good extraction and recovery, and alumina chromatography provided the expected chemical class separations.

KCER surrogate recoveries exceeded the desired 80% level, except for naphthalene-d_8, 4-bromophenol and 4-fluorobiphenyl. Naphthalene also partially eluted in the pentane fraction, rather than being totally in the benzene fraction. This behavior may be due to the volatility of naphthalene and the likely losses due to evaporation during extract concentration.

3.4.1.3 Recovery of water-soluble compounds from fortified sludge. The recoveries of monoethanolamine (MEA), diethanol- amine (DEA), sulfinol and diethylene glycol (DEG) from the ENVIRO-TEST fortified sludge sample from site B are presented in Table 3.6.

Acceptable recoveries of the water soluble compounds from the sludge indicate that the methods were satisfactory for the analyses required.

3.4.1.4 Validation of method for alkanolamine degradation products. Table 3.7 summarizes the recoveries of alkanolamine degradation products from fortified Sample C.

From Table 3.7 it is apparent that the recoveries of alkanolamine degradation products are acceptable. The high level of fortification is a reflection of the poor sensitivity of the Valdez method. The poor sensitivity is likely a result of the small sample sizes which must be used (a maximum sample size of 25 micro- liters). This small sample size is in turn diluted 28 fold during the derivatization step. Hence, concentrations in the order of

Table 3.6. Recoveries of water soluble compounds from sludge B.

Compound	Level of Fortification (µg/g)	Recovery (mean) (%)	Method (detection limit in brackets)
MEA	22.6	82	HPLC (0.2 µg/g)
	226	79	HPLC
DEA	23.2	102	HPLC
	232	103	HPLC
Sulfinol	206	105	GC/FPD (10 µg/g)
DEG	20.4	154	GC/MS (10 µg/g)
	5100	95	silyl - GC/FID (500-600 µg/g)
	40,800	96	silyl - GC/FID

Table 3.7 Recoveries of alkanolamine degradation products from fortified Sample C.

Analyte	Fortification Level (µg/g)	Observed Percent Recovery	
		Replicate 1	Replicate 2
DEA	5800	75	78
HEM	8000	96	92
TEA	5325	69	65
BHEP	4925	78	75
OX	4825	111	113
HEP	6300	102	85

560 µg/mL or greater are needed in the final sample extract. This translates into a detection limit of 9000 µg/g (10 g of sample, 4.0 mL extract) for a sludge sample and 22,400 mg/L for centrifugates. These estimated detection levels could be lowered by a factor of 10 to 100 if gas chromatography/mass spectrometry - selected ion monitoring is employed.

3.4.2 Surrogate Recovery in Centrifugate Sample Analyses

Quality control for all centrifugate analyses was maintained by fortifying the samples with surrogates and noting the recovery. Aqueous samples were analyzed directly for DEA, MEA and DEG, and directly compared to aqueous standards. Sulfinol was found to readily extract into DCM therefore the base/neutral DCM extract was directly analyzed using gas chromatography/flame photometric detection.

Recoveries of the surrogate mix (Table 3.1) analyzed by ENVIRO-TEST are presented in Table 3.8.

Constant and efficient surrogate recovery, as well as elution of the compounds into appropriate fractions, were observed from the centrifugate samples.

3.4.3 Sludge Analyses Results

3.4.3.1 Results of sludge analyses for semi-volatile priority pollutants. A summary of the semi-volatile priority pollutants in the sludge resulting from the initial gas chromatographic screening is presented in Table 3.9.

It was apparent that the major priority pollutants present in the sludge samples were polycyclic aromatic hydrocarbons (PAH), with Site A significantly high in fluorene and Site B high in naphthalene.

Priority pollutant phenolics (acid fraction) were not quantified since the acetylated methanol/water fraction that should contain them revealed little material when the GC/FID analysis was performed.

Table 3.8 Surrogate recovery from centrifugates by ENVIRO-TEST.

Compound	Fraction	Recovery (mean) (%)	RSD[1] (%)
Naphthalene - d_8	benzene	93	66
4 - Bromophenol	methanol/water	70	36
2 - Fluorobiphenyl	benzene	86	15
Dibenzothiophene - d_8	benzene	90	6
Biphenyl - d_{10}	benzene	94	19
Quinoline - d_7	chloroform	100	16
Chrysene - d_{12}	benzene	104	17
Acenaphthalene - d_{10}	benzene	89	11

[1] Relative Standard Deviation; n=3.

Table 3.9. Analysis of sludges for semi-volatile priority pollutants.

Compound	Site	Concentration (µg/g)
Naphthalene	A	93
	B	112
	C	5
Fluorene	A	728
	B	43
	C	ND[1]
Phenanthrene	A	48
	B	44
	C	11

[1] Not detected (detection limit 8 µg/g).

3.4.3.2 Results of sludge analyses for volatile priority pollutants. A summary of benzene, toluene, ethylbenzene and xylene present in the sludges is presented in Table 3.10. The results indicate that the sludges contain very little volatile priority pollutants.

3.4.3.3 Results of sludge analyses for water soluble compounds. The water soluble target compounds present in the sludges are summarized in Table 3.11.

For each site, the predominant water soluble target compound corresponds to the facility gas sweetening method, eg. DEA for Site A.

The KCER analysis of the Site A sludge provides substantially different results than those from ENVIRO-TEST.

3.4.3.4 Analysis of sludge for conventional parameters. Conventional parameters measured in the sludges are presented in Table 3.12.

3.4.3.5 Alkanolamine degradation product results using HPLC/Fluorescence. The presence of degradation products (more correctly primary and secondary amines) are revealed in all sludge and centrifugate samples, as evidenced by the appearance of liquid chromatographic (LC) peaks in the chromatographs. Sludge sample A and centrifugate samples A and C were observed to contain the greatest number of degradation products, as evidenced by complex LC chromatographs. No identification of these observed degradation products was possible other than to compare retention times with authentic reference material. The FMOC esters of the alkanolamine degradation products do not lend themselves to extraction by solvent, hence it is not possible to extract the fluorescent derivatives formed and to analyze them by capillary gas chromatography/mass spectrometry. Identification and confirmation is best achieved by reanalysis using another modified reverse phase LC column.

Table 3.10. BTEX analysis of sludges.

Site	Concentration (µg/g)			
	Benzene	Toluene	Ethylbenzene	Xylene
A	3.1	0.31	0.59	0.04
B	2.0	0.10	0.15	0.04
C	2.6	0.09	0.73	0.04

Table 3.11. Sludge analysis for water soluble target compounds.

Site	Concentration (µg/g)			
	MEA	DEA	Sulfinol	DEG
A	105(6)[1]	5800(77)[1]	92	261
B	ND[2]	22	125	40
C	10	ND	ND	ND

[1] KCER results in brackets.

[2] Not detected: detection limits as follows - MEA/DEA 0.2 µg/g
Sulfinol 10 µg/g
DEG 10 µg/g

Table 3.12. Conventional parameters of sludge samples.

Parameter	Site A	Site B	Site C
Moisture, wt%	31.8	49.9	52.5
Total Sulfur, wt%	4.3	2.9	0.4
Organic Matter, wt%	33.4	17.7	13.6
Total Carbon, wt%	19.4	10.3	7.9
Total Nitrogen, wt%	0.4	0.2	0.2
pH, Filtered water	8.8	8.1	8.0
Electrical Conductivity Filtered Water (mS/cm)	15.7	7.7	11.5
C:N	44.1	54.2	34.3
Phenol (μg/g)	13.5	2.2	0.6
Oil, wt%	17.2	7.8	3.9

3.4.3.6 Alkanolamine degradation products results using GC/FID. GC/FID chromatographs for sludge samples A, B, and C were obtained by silylating an aliquot (25 µL) of concentrated extract (concentrated from 100 mL to 4 mL) using the method of Valdez (1985). In comparing the chromatograms obtained for the samples to the Method Blank, it is apparent that the alkanolamine degradation products are present in sludge sample A. No indication of alkanolamine degradation products was observed for sludge samples B or C. Based on retention time and GC/FID analysis, it was observed that BHEP may be present in sludge sample A. The presence of BHEP was not confirmed by reanalysis using gas chromatography/mass spectrometry (GC/MS).

Centrifugates were not derivatized using the Valdez method or analyzed for alkanolamine degradation products because HPLC/FLUOR analysis revealed that the concentration would not be high enough to warrant detection. For example, the alkanolamine degradation products appearing in the LC chromatograph for centrifugate A were estimated to be 500 ppm. As mentioned earlier, a concentration in excess of 22,000 mg/L (ppm) is required in centrifugates in order to allow for GC/FID and GC/MS identification.

3.4.3.7 Characterization of sludges using gas chromatography/mass spectrometry. After general screening of all fractions using GC/FID, only the fractions showing more complex GC peaks were subjected to GC/FPD, GC/NPD and GC/MS analyses.

When computerized identification was not possible (e.g. due to co-elution of components) the laboratories resorted to manual interpretation using first principles. Confidence intervals were assigned as follows to indicate the level of accuracy of the identification:

Number 1: Very confident, clean spectra, excellent match with library spectra.

Number 2: Some small spectra differences with library match. A good comparision. Reference standard needed for positive identification.

Number 3: Possible compound or very similar to. Spectra is contaminated. Reference standard needed for positive identification.

Number 4: Evidence of possible structure and molecular weight. Compound not identified.

Number 5: Unable to identify.

These confidence indicators are presented in brackets beside the concentrations of the components in the following tables.

The general GC/MS characterizations of the three sludge samples as evaluated by ENVIRO-TEST are summarized in Tables 3.13 to 3.15. The specific compounds identified by ENVIRO-TEST in all fractions analyzed are presented in Tables 3.16 to 3.18 and include the KCER evaluation for comparison.

3.4.4 Centrifugate Analyses Results

3.4.4.1 Results of centrifugate analyses for semi-volatile priority pollutants. A summary of the semi-volatile priority pollutants in the centrifugate as a result of the initial gas chromatographic screening is presented in Table 3.19. Site A generally contains the highest levels of semivolatile priority pollutants, with fluorene being the most dominant compound.

3.4.4.2 Results of centrifugate analyses for volatile priority pollutants. A summary of benzene, toluene, ethylbenzene and xylene present in the centrifugate samples is presented in Table 3.20.

Centrifugate from Site A contains high levels of toluene, ethylbenzene and benzene. In the Site C centrifugate, noticeable levels of toluene and xylene are seen. Site B contains very little BTEX.

3.4.4.3 Results of centrifugate analyses for water soluble compounds. The water soluble target compounds present in the centrifugates are summarized in Table 3.21.

Table 3.13. General GC/MS characterization of Site A sludge.

Fraction	Compound	Approximate Concentration (μg/g)	Relative Percent (%)
Hexane	C_3 benzenes	111	1
	Saturates, C_{10}-C_{25}	3421	36
	Substituted Tricyclic, aliphatic compounds	5929	63
Benzene	Substituted naphthalenes	377	3
	Substituted fluorenes	1362	11
	Substituted dibenzothiophenes	6766	56
	Substituted polycyclic, aromatic sulfur heterocycles	2979	25
Chloroform	Dibenzothiophene	432	19
	PANH, MW 264	571	25
	PANH, MW 308	1317	57
Methanol Extract after DCM extract	Substituted dibenzothiophene	81	49
	Organic acids	21	13
	Unknown, N-containing	50	30
	Substituted fluorene	8	5
	Unknown, MW 168 & 182	4	2

Table 3.14. General GC/MS characterization of Site B sludge.

Fraction	Compound	Approximate Concentration (µg/g)	Relative Percent (%)
Hexane	C_3 benzenes	150	2
	Saturates, C_{10}-C_{29}	8131	90
	Methyl-tetrahydronaphthalene	137	2
	Dimethyl tetralin	128	1
	S_8 (molecular sulfur)	164	2
	Unsaturates, olefins	370	4
Benzene	C_4 benzene	77	1
	Substituted naphthalene	2787	58
	Substituted fluorene	113	2
	Unknowns	119	2
	Substituted dibenzothiophenes	1407	29
	Substituted phenanthrene	118	2
	Dimethyl benzothiophene	44	1
	PASH	97	2
	S_8	118	2
Chloroform	Unknown, MW 120 & 220	444	43
	Phthalates	422	41
	S_8	174	17
Methanol Extract after DCM extraction	Substituted naphthalene	23	41
	Substituted dibenzothiophene	26	46
	Organic acid	5	9
	PASH	3	5

Table 3.15. General GC/MS characterization of Site C sludge.

Fraction	Compound	Approximate Concentration (μg/g)	Relative Percent (%)
Hexane	Saturates, C_{10}-C_{30}	6055	96
	Unsaturated alkenes, C_{14}-C_{36}	225	4
Benzene	C_4 benzene	63	3
	Saturates, C_{10}-C_{26}	570	27
	1-H indene, 2,3-dihydro - 4,7-dimethyl	13	1
	Substituted naphthalene	715	34
	Substituted fluorene	186	9
	Substituted dibenzothiophene	352	17
	Substituted phenanthrene	220	10
Methanol Extract after DCM Extraction	Organic Acids	13	62
	Substituted dibenzothiophene	1	5
	Unknowns	7	33

Table 3.16. GC/MS specific compound identification for Site A sludge.

Fraction	Compound	Concentration (μg/g) (interpretation confidence level in brackets)	
		ENVIRO-TEST	KCER
Hexane	Trimethyl benzene	111 (1)	-
	Saturates, C_{10}-C_{25}	3421 (1)	7460 (1)
	Aliphatic disulfides	-	333 (3)
	Tetralins	-	4313 (3)
	Dibenzothiophene	-	282 (1)
Benzene	Methylnaphthalene	233 (1)	262 (1)
	Dimethylnaphthalene	146 (1)	
	Fluorene	649 (1)	1184 (1)
	Methylfluorene	724 (1)	
	Dibenzothiophene	2972 (1)	12100 (1)
	Methyldibenzothiophene	2724 (1)	
	Dimethyldibenzothiophene	651 (1)	
	Ethyldibenzothiophene	419 (1)	
	Diphenylamine	-	32 (1)
	Aliphatic trisulfides	-	256 (3)
Chloroform	Dibenzothiophene	432 (1)	38 (1)
	Unknown, N-containing MW 264 & 308	1888 (5)	-
	Diphenylamine	-	6 (1)
	$C_{18} H_{34}$	-	224 (2)
	Aliphatic di-or trisulfides	-	90 (4)
Methanol-Water	Dibenzothiophene	-	8 (1)
	Diphenylamine	-	2 (1)
	Aliphatic trisulfides	-	3 (3)
Methanol Extract of Sludge, after DCM Extraction	Dibenzothiophene	35 (1)	2 (1)
	Methyldibenzothiophene	37 (1)	-
	Dimethyldibenzothiophene	8 (1)	-
	Fluorene	5 (1)	8 (1)
	Methylfluorene	5 (1)	-
	Unsaturated acids	21 (4)	-
	Unknown, N-containing MW 168 & 182	54 (5)	-
	MEA	-	6 (1)
	DEA	-	77 (1)
	TEA	-	2 (1)
	Diphenylamine	-	2 (1)
	Honanoic acid	-	1 (1)
	Hexadecanoic acid	-	7 (1)
	Aliphatic trisulfides	-	3 (3)

Table 3.17 GC/MS Specific Compound Identification for Site B Sludge

Fraction	Compound	Concentration (μg/g) (interpretation confidence level in brackets)
Hexane	Ethyl-methylbenzene or isopropylbenzene	105 (1)
	Trimethylbenzene	44 (1)
	Olefin, MW 196	150 (1)
	MW 226	47 (1)
	MW 230	172 (1)
Benzene	Naphthalene	184 (1)
	Methylnaphthalene	636 (1)
	Dimethylnaphthalene	1041 (1)
	Ethylnaphthalene	165 (1)
	Ethylmethylnaphthalene	71 (1)
	Trimethylnaphthalene	646 (1)
	Fluorene	12 (1)
	Methylfluorene	101 (1)
	Dibenzothiophene	741 (1)
	Methyldibenzothiophene	666 (1)
	Phenanthrene	55 (1)
	Dimethylphenanthrene	63 (1)
	PASH, MW 206	81 (2)
Chloroform	Unknown, MW 120 & 220	444 (5)
	Phthalate	422 (1)
	S_8	174 (1)
Methanol Extract after DCM Extraction	Methylnaphthalene	9 (1)
	Dimethylnaphthalene	9 (1)
	Trimethylnaphthalene	2 (1)
	Ethyl-methylnaphthalene	1 (1)
	1-(2-propenyl) naphthalene	2 (1)
	Dibenzothiophene	17 (1)
	Methyldibenzothiophene	8 (1)
	Acid, MW 156	5 (4)
	PASH, MW 346	3 (4)

Table 3.18 GC/MS specific compound identification for Site C sludge.

Fraction	Compound	Concentration (μg/g) (interpretation confidence level in brackets)
Hexane	Saturates, C_{10}-C_{30}	6055 (1)
	Unsaturated alkenes, C_{14}-C_{16}	225 (4)
Benzene	Methylnaphthalene	173 (1)
	Dimethylnaphthalene	116 (1)
	Ethylnaphthalene	66 (1)
	Ethylmethylnaphthalene	47 (1)
	Trimethylnaphthalene	186 (1)
	Tetramethylnaphthalene	126 (1)
	Fluorene	33 (1)
	Methylfluorene	40 (1)
	Dimethylfluorene	113 (1)
	Methyldibenzothiophene	103 (1)
	Dimethyldibenzothiophene	174 (1)
	Ethyldibenzothiophene	19 (1)
	Trimethyldibenzothiophene	56 (1)
	Methylphenanthrene	73 (1)
	Dimethylphenanthrene	86 (1)
	Trimethylphenanthrene	61 (1)
Methanol Extract after DCM Extraction	Organic acids	13 (5)
	Substituted dibenzothiophene	1 (1)

Table 3.19. Analysis of centrifugate samples for semi-volatile priority pollutants.

Compound	Site	Concentration (mg/L)
Naphthalene	A	44
	B	ND[1]
	C	1.5
Fluorene	A	400
	B	ND
	C	1.5
Phenanthrene	A	26
	B	ND
	C	2.5
Anthracene	A	ND
	B	ND
	C	ND
Bis(2-ethylhexyl) phthalate	A	18
	B	ND
	C	0.50
Diethyl phthalate	A	ND
	B	0.88
	C	ND
Dibutyl phthalate	A	ND
	B	ND
	C	0.22

[1] Not detected: detection limit for Sample A is 0.80 mg/L and for Samples B and C is 0.08 mg/L.

Table 3.20. BTEX analysis of centrifugates.

Site	Concentration (mg/L)			
	Benzene	Toluene	Ethylbenzene	Xylene
A	409	755	39	ND[1]
B	ND	0.07	0.16	1.7
C	6.9	124	8.4	90

[1] Not detected: detection limit is 0.01 mg/L.

Table 3.21. Centrifugate analysis for water soluble target compounds.

Site	Concentration (mg/L)			
	MEA	DEA	Sulfinol	DEG
A	3.2	12 624	1 590	1 280
B	0.6	0.2	47 658	ND[1]
C	0.8	0.6	ND	ND

[1] Not detected: detection limits are as follows -

MEA/DEA	0.2 mg/L
Sulfinol	0.01 mg/L
DEG	10 mg/L

For Sites A and B, the levels of water soluble compounds clearly suggest the gas plant type by the distinctly high levels of the appropriate water soluble compounds, i.e., DEA for Site A and sulfinol for Site B. Site C levels are too low for a reliable relationship with gas plant type.

KCER GC/MS analysis of the Site C centrifugate did not detect any water soluble compounds.

3.4.4.4 Analysis of centrifugate for conventional parameters. Table 3.22 presents the centrifugates' conventional parameters.

3.4.4.5 Characterization of centrifugates using gas chromatography/mass spectrometry. Analysis of the centrifugate samples was performed according to the sample screening procedure presented for sludges, as discussed in Section 3.4.3.7.

The general GC/MS centrifugate characterizations are presented in Tables 3.23 to 3.25, and detailed GC/MS characterizations of the three centrifugate samples are summarized in Tables 3.26 to 3.28, with the specific compounds identified in all fractions.

3.5 DISCUSSION OF RESULTS

3.5.1 Comparison of ENVIRO-TEST Sludge and Centrifugate Results

The summary of results of the ENVIRO-TEST general GC chemical analyses of the sludge and centrifugate fractions are presented in Table 3.29. Comparison of the total fractions and chemical classes present in the sludge and the centrifugate fractions show similar trends, with a few exceptions.

No compounds were detected in the sludge acid fraction nor in the centrifugate methanol/water fraction. Weak acids are expected in the methanol/water fraction, as is supported by the recovery of the surrogate 4-bromophenol, while more acids would require extraction

Table 3.22. Conventional parameters for centrifugate samples.

Parameter	Site A	Site B	Site C
Electrical Conductivity(mS/cm)	5.3	8.7	18.0
Nitrate/Nitrite N (mg/L)	LT[1] 0.05	LT 0.05	LT 0.05
Ammonia N (mg/L)	61.6	9.2	46.6
TKN Nitrogen (mg/L)	66.4	10.7	53.6
Total Organic Carbon (mg/L)	4030	1560	3850
Phenols (mg/L)	0.90	1.30	1.02
Sulfides (mg/L)	0.05	0.35	0.05

[1] Less than

Table 3.23. General GC/MS characterization of Site A centrifugate.

Fraction	Compound	Approximate Concentration (mg/L)	Relative Percent (%)
Hexane	C_3 benzenes	210	1
	Saturates, C_{10}-C_{22}	8 835	51
	Substituted tricyclic aliphatic compounds	3 570	21
	Substituted polycyclic aliphatic compounds	3 300	19
	Molecular sulphur, S_8	435	3
	Unknown	840	5
Benzene	Substituted fluorene	2 055	12
	Substituted dibenzothiophene	13 350	75
	Polycyclic aromatic sulphur heterocycles	2 355	13
Chloroform	Alcohols	675	63
	Cyclic acids	285	27
	Phthalates	60	6
	Polycyclic aromatic sulphur heterocycles	45	4
Acid	Saturates, C_{10}-C_{19}	1 463	32
	Substituted fluorene	142	3
	Substituted dibenzothiophene	1 388	30
	Substituted tricyclic aliphatics	243	5
	Substituted cyclic aliphatics	641	14
	Polycyclic aromatic sulphur heterocycles	448	10
	Molecular sulphur, S_8	90	2
	Polycyclic aromatic hydrocarbons	72	2
	Substituted phenols	142	3

Table 3.24. General GC/MS characterization of Site B centrifugate.

Fraction	Compound	Approximate Concentration (µg/L)	Relative Percent (%)
Hexane	Saturates	28	100
Benzene	Substituted naphthalene	62	100
Chloroform	Alcohols	2 653	41
	N-containing compounds	1 783	27
	N- and S-containing compounds	2 037	31
	Phthalates	69	1
Acid	Organic acids	646	15
	Sulpholane	249	6
	Unsaturated acids	817	19
	Phthalates	1 183	27
	N-containing compounds	1 413	33

Table 3.25. General GC/MS characterization of Site C centrifugate.

Fraction	Compound	Approximate Concentration (mg/L)	Relative Percent (%)
Hexane	Saturates, C_{10}-C_{26}	2 200	98
	Unsaturated olefins	40	2
Benzene	Ethyldimethylbenzene	2	1
	Substituted naphthalene	82	50
	Substituted fluorene	10	6
	Substituted phenanthrene and/or anthracene	28	17
	Saturates, C_{15}-C_{22}	12	7
	Substituted dibenzothiophene	31	19
Chloroform	Long chain unsaturated alcohols	4	33
	Phthalates	8	67
Acid	Saturates, C_{10}-C_{25}	80	99
	Phthalates	1	1

Table 3.26. GC/MS specific compound identification for Site A centrifugate.

Fraction	Compound	Concentration (mg/L) (interpretation confidence level in brackets)
Hexane	Saturates, C_{10}-C_{22}	8835 (1)
	Trimethylbenzene	210 (1)
	Substituted tricyclic compounds, MW 136 to 190	3570 (4)
	Substituted polycyclic aliphatic compounds, MW 202 to 230	3165 (5)
	Substituted polycyclic aliphatic compounds, MW 200 to 214	135 (5)
	S_8 (molecular sulfur)	435 (1)
	Unknowns, MW 176, 190 and 240	840 (5)
Benzene	Fluorene	870 (1)
	Methylfluorene	1170 (1)
	Dibenzothiophene	8655 (1)
	Methyldibenzothiophene	3450 (1)
	Dimethyldibenzothiophene	795 (1)
	Ethyldibenzothiophene	450 (1)
	PASHs	2355 (4)
Chloroform	Saturated alcohols	105 (4)
	Cyclic alcohols	225 (5)
	Unsaturated alcohols	345 (5)
	Unsaturated acids, cyclic	285 (5)
	Phthalates	60 (1)
	PASHs	45 (4)
Acid	Saturates, C_{10}-C_{19}	1463 (1)
	Substituted fluorenes	142 (1)
	Dibenzothiophene	831 (1)
	Methyldibenzothiophene	557 (1)
	Substituted tricyclic aliphatics	243 (5)
	Substituted cyclic aliphatics	641 (5)
	S_8 (molecular sulfur)	90 (1)
	PAH	72 (4)
	Ethyldimethylphenol	142 (1)

Table 3.27. GC/MS specific compound identification for Site B centrifugate.

Fraction	Compound	Concentration (µg/L) (interpretation confidence level in brackets)
Hexane	Saturates	28 (1)
Benzene	Substituted naphthalene, C_2	62 (1)
Chloroform	Saturated alcohol	669 (5)
	Unsaturated alcohol	1984 (5)
	N-containing compounds	1783 (4)
	N- and S- containing compounds	2037 (4)
	Phthalates	69 (1)
Acid	Organic acids	646 (5)
	Sulpholane	249 (1)
	Unsaturated acids	817 (4)
	Phthalates	1183 (1)
	N-containing compounds	1413 (5)

Table 3.28. GC/MS specific compound identification for Site C centrifugate.

Fraction	Compound	Concentration (mg/L) (interpretation confidence level in brackets)	
		ENVIRO-TEST	KCER
Hexane	Saturates, C_{10}-C_{26}	2200 (1)	139 (1)
	Olefins	40 (4)	-
	Tetramethylbenzene	-	1 (1)
	Alicyclic hydrocarbons	-	0.5 (1)
Benzene	Methylnaphthalene	11 (1)	
	Dimethylnaphthalene	29 (1)	7 (1)
	Ethylnaphthalene	6 (1)	
	Trimethylnaphthalene	23 (1)	
	Ethylmethyl - or isopropylnaphthalene	9 (1)	-
	Tetramethylnaphthalene	3 (1)	-
	Fluorene	1 (1)	
	Methylfluorene	3 (1)	2 (1)
	Dimethylfluorene	5 (1)	
	Trimethylfluorene	1 (1)	
	Phenanthrene	3 (1)	
	Methylphenanthrene/anthracene	9 (1)	3 (1)
	Dimethylphenanthrene/ anthracene	8 (1)	
	Trimethylphenanthrene/ anthracene	5 (1)	
	Tetramethylphenanthrene/ anthracene	2 (1)	
	Methyldibenzothiophene	10 (1)	
	Dimethyldibenzothiophene	11 (1)	
	Ethyldibenzothiophene	4 (1)	7 (1)
	Ethylmethyl - or isopropyldibenzothiophene	2 (1)	
	Trimethyldibenzothiophene	3 (1)	
	Biphenyl (mono & dimethyl)	-	0.04 (1)
	Diphenylmethane (dimethyl)	-	0.2 (1)

... continued

Table 3.28. GC/MS specific compound identification for Site C centrifugate (concluded).

Fraction	Compound	Concentration (mg/L) (interpretation confidence level in brackets)	
		Enviro-Test	KCER
Chloroform	Long chain unsaturated alcohol	4 (5)	-
	Phthalate	8 (1)	-
	Phenol	-	0.2 (2)
	Tolylethanol	-	0.1 (2)
	Methoxytoluene	-	0.03 (2)
	Methoxy benzoic acid	-	0.2 (1)
	Trimethylindan	-	0.06 (3)
	Tetramethylindene	-	0.02 (3)
	Trimethylindole	-	0.004 (3)
	Diphenylamine	-	0.02 (1)
	Carbazoles	-	0.4 (1)
Methanol:Water	Benzoic acid	-	0.02 (1)
	N-acetyldiethylamine	-	0.01 (1)
	N-acetylcyclohexylamine	-	0.1 (1)
	Diphenylamine	-	0.02 (1)
Acid	Saturates, C_{10}-C_{25}	80 (1)	50 (1)
	Phthalates	1 (1)	-
	Benzenes	-	0.9 (1)
	Tetralins	-	1.1 (3)
	Naphthalenes	-	1.8 (1)
	Dimethylphenanthrene	-	0.4 (1)
	Methylbenzoic acid	-	7.6 (1)

Table 3.29. Summary of ENVIRO-TEST general GC chemical results.

Parameter		Sludge Concentration (µg/g)			Centrifugate Concentration (mg/L)		
		Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
Total Fractions[1]:	Hexane	9461[2] (3)	9080 (6)	6280 (2)	17190 (7)	LT1 (1)	2240 (2)
	Benzene	11484 (4)	4880 (9)	2119 (7)	27745 (7)	LT1 (1)	147 (20)
	Chloroform	2320 (3)	1040 (3)	-	1065 (6)	6 (5)	12 (2)
	Methanol/Water	164 (5)	57 (4)	21 (3)	-	-	-
	Acid	-	-	-	4181 (9)	4 (5)	81 (2)
Chemical Classes:	Saturates	3421	-	6055	8835	LT1	2200
	PAH/PASH/PANH	7657	4398	1287	2472	-	138
	Phenols	-	-	-	142	-	-
	BTEX	4	2	3	1203	2	229
	Water Solubles:						
	MEA	105	-	10	3	LT1	LT1
	DEA	5800	22	-	12624	LT1	LT1
	Sulfinol	92	125	-	1590	47658	-
	DEG	261	40	-	1283	-	-

[1] Total fraction concentration being the summation of components (numbers of chemical families in brackets) found in ENVIRO-TEST general GC/MS Characterization.

[2] The highest value of the three samples is underlined.

with more polar solvents. The preliminary analysis of the acetylated sludge methanol/water fraction revealed little material and was not analyzed further by GC/MS.

Comparison of the fractions generated by each sample shows that Sample C centrifugate contained compounds extractable by chloroform but the sludge did not. In addition, the chloroform fraction of the other sludges contained PANH's, phthalates and elemental sulphur, while the chloroform fraction of the Sample C centrifugate contained low levels of unsaturated alcohols and phthalates.

Each of the chemical classes are generally more prevalent in Sample A (sludge and centrifugate samples). An exception is the sulfinol, which is more prevalent in Sample B sludge and centrifugate (the sulfinol plant site sample). Another exception is observed with the saturates, with Sample C sludge having the highest concentration, followed by Sample A and then Sample B sludges.

3.5.2 Evaluation of Volatile and Semi-Volatile Pollutant Analysis Method Performance

The results of the analyses indicate that the goal of developing an effective analytical method for the determination of the presence of volatile and semi-volatile priority pollutants in gas plant sludge and centrifugate samples was achieved. The procedures presented provide simple, straightforward and accepted methodologies to categorize the complex mixture of chemicals that may be present in the gas plant samples. The alumina column fractionation steps used to generate the chemical class separation are routinely performed to permit quantification and to minimize chemical interactions and interferences. The screening fractions, however, only permit a rough analytical segregation of the general groups of compounds without precise evaluation. More specific identification of component chemicals is provided by GC/MS evaluation.

Comparison of the GC/MS results from ENVIRO-TEST to those of KCER, however, indicate considerable difficulty with the duplication of the analysis. Review of the specific compound identifications for Site A sludge (Table 3.16) and Site C centrifugate (Table 3.28) presents the variation in the compounds identified. KCER identification utilized a number of composite groupings while ENVIRO-TEST identified individual components. KCER also identified a different selection of components than ENVIRO-TEST did. KCER also analyzed for MEA and DEA using GC/MS, noting their presence in the Site A sludge. The predominance of DEA correlates well with the Site A being a DEA gas plant.

Comparison of the Site A sludge GC/MS total ion chromatograms from KCER and ENVIRO-TEST indicates that all KCER fraction chromatograms are dissimilar to the ENVIRO-TEST results, except for the benzene fraction. For the pentane and benzene fractions, ENVIRO-TEST observed more peaks than KCER and, for the chloroform fraction, KCER observed more peaks. The acetylated methanol fraction analysis resulted in approximately the same number of peaks but with a different profile.

Centrifugate C GC/MS total ion chromatograms comparison between ENVIRO-TEST and KCER indicates that the pentane and acid fractions presented similar chromatograms but the others are dissimilar. The benzene and the acetylated methanol fractions both showed different profiles while the KCER chloroform fraction presented more peaks.

Partial explanation of the variability between the laboratories may come from a review of the recoveries of the alumina-calibrated compounds (Table 3.4) and the recoveries of the surrogate from the sludge (Table 3.5). It is apparent that KCER experienced more difficulty with the performance of their column, as can be seen by the recovery variability and the splitting of some calibration compounds into more than one fraction, indicating improper fractionation. The prevalence of significant differences in the comparison of fraction GC/MS total ion chromatograms also suggests that different extracts were analyzed. Since a number of chromatograms are different, it would be expected that different compound identifications would occur. These differences indicate that problems existed with sample extraction capabilities or with sample heterogeneity.

In the context of environmental impact determination for gas plant sludges, it will be necessary to eliminate the sources of this variation.

3.5.3 Performance of the Water-Soluble Analysis Method

Following a review of available literature on methods for determining the water soluble components in gas plant sludges and centrifugates, ENVIRO-TEST presented and applied an amine derivatization method derived from Nasholm et al (1987) and Einarsson et al (1986). This method, as presented in Section 3.2.1.3, was utilized to identify monoethanolamine (MEA), diethanolamine (DEA). Diethylene glycol (DEG) and sulfinol were identified using GC/MS and GC/FPD, respectively, on the methanol extract of the sample.

The results of the analysis for the water-soluble target compounds in the sludges (Table 3.11) and in the centrifugates (Table 3.21) successfully identify the source of the samples, i.e., Site A being DEA, Site B being sulfinol and Site C being MEA. Identification for DEA and sulfinol was very clearly defined by the order of magnitude difference in alternate component concentrations. With MEA, the concentration trend was present but not to the extent occurring with DEA and Sulfinol.

3.5.4 Alkanolamine Degradation Products Evaluation

Analysis of centrifugate and sludge extracts by HPLC revealed the presence of alkanolamine degradation products. Although sensitive for screening samples for primary and secondary alkanolamines and associated degradation products, this method is inappropriate for providing confirmatory proof of the presence or absence of such products in complex samples. Reanalysis using another

HPLC column is the best level of confirmation which can be achieved. However, the method is extremely sensitive and has merit for monitoring purposes.

Analysis of concentrated sludge extracts by GC/FID after derivatization using the Valdez (1985) method revealed the presence of degradation products including BHEP by GC/FID analysis. The presence of this degradation product was not confirmed by GC/MS analysis. The presence of BHEP in sludge A is consistent with the past experiences of ENVIRO-TEST evaluations of reclaimed amine samples taken from DEA gas sweetening plants. However, TEA, BHEED and THEED were also expected to be present at much higher levels than BHEP and this was not observed. Although the Valdez (1985) method followed by GC/MS analysis is ideally suited for alkanolamine degradation products identification and confirmation, this method is not very sensitive. Detection levels are too high for this method to be considered useful for environmental monitoring. Clearly another method is required which allows for the detection of alkanolamine degradation products in samples of centifugate and sludge at low levels (PPM) and which is amenable to GC/MS confirmation.

A recent publication by Valdez and Reier (1986) suggests that derivatization of alcohols with 3,5-dinitrobenzoyl chloride forms a derivative which is responsive to HPLC/UV detection and which can be extracted with a solvent and analyzed by gas chromatography. Since alkanolamine degradation products are alcohols, the potential of this method should be explored.

3.5.5 Inter-Laboratory Variability

A study objective had been established to test the reproducibility of the organic analyses by submitting duplicate samples of a sludge (Site A) and a centrifugate (Site C) to a designated laboratory (KCER) and to compare the results of the two laboratories. As mentioned in Section 3.5.2, comparison of the volatile and semi-volatile pollutant analyses indicate considerable lack of agreement between laboratories. This variation occurred in spite of the close interaction between laboratories during the study. Though experienced with analytical methodologies and, considering the extensive efforts to distribute a representative sample, the laboratories indicated obvious differences in specific composition and in the ability to replicate recoveries of known standards. As previously noted (Table 3.4), the discrepancies between participants are not only with recovery levels but also with the fractions in which certain standards appear. These results lead to questioning the validity of KCER's alumina column preparation.

The methods utilized by Enviro-Test are generally considered to be simple and, to a large extent, standard procedures and should easily be adopted by laboratories involved in environmental assessments. Comparison of the two sets of results, however, indicates that problems existed with either the sample extraction or fractionation methodology so that reproducibility of results is highly questioned. Problems may also have existed with sample homogeneity. It should be noted, however, that a judgement cannot be made of the validity of the method by only one other laboratory duplicating analysis of only a portion of the entire program. If one wishes to "ruggedness-test" the method, testing similar to the toxicity tests (Section 4.0) should be performed where four to five laboratories repeat, at least in triplicate, the analyses.

3.5.6 Cost Effectiveness of Proposed Organic Chemical Fractionation Method

This organic chemical analysis protocol fractionates complex samples into chemical classes to which environmental concerns can be associated, eg. PANH's, PASH's or phenols. Conventional methods of complex sludge analysis, such as "dilute and shoot", generally identify saturates and are based on an assumption that the diluted response will relate to the original concentration. Losses on dilution may occur of the less concentrated compounds as a consequence of detection limitations of the procedures. Thus one should fractionate the original complex samples to assist in identifying all toxicologically significant compounds.

The information obtained from the GC/FID analysis and a mass balance enable one to choose which fraction to evaluate, if needed, with the more costly GC/MS analysis. The potential cost-savings due to elimination of GC/MS characterization as a result of this fractionation GC/FID preliminary screening is close to $1000 per sample, a cost saving of approximately 50%.

As an example, the GC/FID, GC/FPD analyses demonstrated that the benzene fraction contained primarily PASH's. This was confirmed by GC/MS analysis, which also confirmed that the PAH's were confined to the same fraction. Using fractionation along with GC/FID, GC/FPD and GC/NPD, one can economically determine where PASH and PANH are concentrated. With this low-cost data, one can prioritize GC/MS requirements.

4. TOXICITY TESTING

Toxicity data is easily quantified. Toxicity testing can demonstrate a degree of reproducibility comparable to that of chemical analyses and QA/QC programs similar to those implemented in chemical laboratories can be put in place for toxicity testing programs. The major difference between toxicity and chemical testing is that toxicity tests can measure the hazard of a sample to living organisms, e.g. they demonstrate the effect of sample exposure to organisms which cannot be accurately predicted from chemical data alone. Toxicity tests integrate the effects of exposure of biological organisms to all the constituents of a sample. The response measured most often in acute (short-term) tests is lethality (death). However, sublethal responses such as inhibition of movement, enzyme activity or light production inhibition can also be used as a measure of effect in acute toxicity testing.

The objective of this study component was to assess the applicability of selected aquatic and terrestrial acute toxicity tests in providing a measure of the environmental significance of gas plant sludges with specific emphasis on test reproducibility, cost-effectiveness and the overall comprehensiveness of the battery of tests approach.

The specific objectives of this portion of the study were to:

- verify the precision and reproducibility of three aquatic toxicity tests for estimating the environmental significance of interstitial liquids centrifuged from natural gas plant waste sludges; and

- assess the performance and reproducibility of two terrestrial toxicity tests in estimating the environmental significance of whole sludge samples applied to soils.

The battery of aquatic toxicity tests conducted included:

- an acute lethality/immobility test, of 48-hour duration, using the cladoceran Daphnia magna;
- an acute sublethal growth inhibition test, of 96-hour duration, using the green unicellular algae Selenastrum capricornutum;
- an acute sublethality bioluminescence inhibition test (Microtox), of 5 and 15-minute durations, using the bacterium Photobacterium phosphoreum.

The terrestrial toxicity tests conducted included:

- a sublethal seed germination soil test (Thomas and Cline, 1985) utilizing two plant species, barley (Hordeum vulgare) and cucumber (Cucumis sativa), for a duration of seven days; and
- an earthworm lethality test of 14-day duration utilizing Eisenia andrei as the test species.

The aquatic acute lethality/sublethality toxicity tests were chosen as a result of the findings of the previous study (Phase II), where a larger battery of aquatic toxicity tests were evaluated. In the Phase II study, a seed germination test was the only terrestrial toxicity test evaluated. The earthworm lethality test and a modified seed germination test were evaluated in the current study to improve upon the terrestrial toxicity test results of the Phase II study. It was also recommended in the Phase II study that centrifugate analysis and testing was preferred over laboratory-generated leachate analysis, and that 95% confidence intervals must be calculated and reported.

When interpreted in combination with the chemical analyses, the aquatic and terrestrial toxicity tests may provide a broad range of site toxicity information for site characterization of hazardous materials and indicate the extent of contamination by the waste material.

Organic analyses and toxicity tests were conducted on the same centrifugate and whole sludge samples. Each test was performed in triplicate since this is the minimum number of data points that can be used to perform linear regression analysis of the data.Negative controls (100 percent clean dilution water) were performed during each test. Standardization of the toxicity test procedure at each laboratory was accomplished by toxicological assessment of two reference toxicants, zinc chloride and 2-chloroacetamide. The reference toxicants were prepared at set concentrations and distributed by a central facility (WESTERN RESEARCH). An exception to this was EPA, who prepared their own reference toxicant solutions. Initial range finding toxicity tests were performed using broad concentration ranges to narrow the critical concentration range for each test organism and reference toxicant. Definitive toxicity tests were performed at concentrations around the LC_{50} or EC_{50}, using reference toxicant concentrations that provided for greater resolution of the test endpoints.

The samples of sludge centrifugate, utilized for the aquatic toxicity tests, were generated as explained in Section 2. A discussion of the terrestrial toxicity test sludge sample homogenization and subsampling procedure is also provided in Section 2. Every effort was made to rapidly transport the samples to the participating laboratories to prevent or minimize sample degradation and chemical constituent modification.

To minimize the variation resulting from the use of different test organism stock cultures, the test organisms for each toxicity test were distributed to all laboratories from a common source. An exception to this was that EPA did not supply BIOQUEST with earthworms, as they died in transit. Slight changes in procedure also may cause significant variations in the results; therefore, extensive communications among all test participants and the project manager were required to standardize procedures of testing and methods of reporting, as well as attempt to reduce misinterpretation of written and oral instructions. The timing of the test performance by laboratories was strictly controlled so that toxicity testing was initiated in all participating laboratories on the same day.

This section briefly describes the toxicity test procedures (detailed description of procedures is found in Appendix B); discusses the statistical methods used to analyze the results; summarizes the test results (presented as mean, standard deviation and coefficient of variation for each triplicate test); and discusses the significant patterns observed. The presentation will be on a test-by-test basis.

4.1 PROCEDURE STANDARDIZATION USING REFERENCE TOXICANTS

WESTERN RESEARCH handled the distribution of the reference toxicants (zinc chloride at a concentration of 3700 mg/L as zinc and 2-chloroacetamide at a concentration of 5000 mg/L) to all laboratories with the exception of EPA. The choice of the inorganic and organic reference toxicants was based on the advice of toxicologists from the Hazardous Waste Assessment Team, U.S. EPA, Corvallis, Oregon. The solutions were distributed and the results of the aquatic and terrestrial toxicity tests are presented in Tables 4.1 and 4.2, respectively. Statistical analysis of result variation was not done.

From these reference toxicant results, an apparent problem with the algae test performed by BIOQUEST was observed (see Table 4.1). Due to the degree of the inequity and the inability to resolve this difference after two repeat attempts, the Technical Steering Committee decided that BIOQUEST would not be asked to undertake any further algae toxicity testing during this project. Another discrepancy, noted on Table 4.1 with BETA's Microtox zinc chloride EC_{50} (5 min) value, can be explained by the high variability in the result, as indicated by its large standard deviation. Review of

Table 4.1. Aquatic toxicity test reference toxicant results, as mean LC_{50} or EC_{50} (mg/L).

Test	Laboratory	Zinc Chloride	2-Chloroacetamide
Daphnia (LC_{50})	EC (Edm)	0.79 (0.43-1.15)[1]	17.25 (15.99-18.51)
	BIOQUEST	3.31 (2.59-4.05)	10.94 (4.16-17.72)
	EPA	1.30 - [2]	10.96[3] (4.18-20.20)
Algae (EC_{50})	EC (Edm)	0.04 (0.02-0.06)	21.30 (21.3-21.3)
	BIOQUEST	1.18 (1.02-1.34)	37.47 (35.07-39.87)
	EPA[2]	0.05[4] (0.04-0.06)	13.42[4] (9.06-17.78)
Microtox - 5 Minute (EC_{50})	BETA	75.17 (0-162.31)	2430.00 (2226.04-2633.96)
	EC (Edm)	8.73 (7.09-10.37)	2459.20 (2215.74-2702.66)
	ESSO	5.48 (4.92-6.04)	2900.30 (2229.10-3571.50)
	ERCB	6.37 (1.49-11.25)	2633.33 (1941.99-3324.67)
- 15 Minute (EC_{50})	BETA	3.75 (2.61-4.89)	913.33 (888.39-938.27)
	EC (Edm)	2.30 (1.98-2.62)	881.93 (872.99-890.87)
	ESSO	1.04 (0.58-1.50)	978.47 (870.77-1086.17)
	ERCB	1.43 (0.75-2.11)	921.67 (589.57-1253.77)

---

[1] 95% confidence intervals generated from provided standard deviations (mean ± 2 standard deviations)(Hamilton et al., 1977).

[2] EPA provided no standard deviations.

[3] Average of six toxicity tests.

[4] Average of five toxicity tests.

Table 4.2. Terrestrial toxicity test reference toxicant results, as mean LC_{50} or EC_{50} (mg/kg).

Test	Laboratory	Zinc Chloride	2-Chloroacetamide
Seed Germination - Barley (EC_{50})	BETA	N.E.[1]	89.94 (78.02 - 103.69)[2]
	AEC	N.E.	55.31 (47.70 - 64.13)[2]
	EPA	N.E.	7.03 (5.50 - 8.90)
Seed Germination - cucumber (EC_{50})	BETA	N.E.	100.41 (89.55 - 112.59)[2]
	AEC	N.E.	99.61 (92.56 - 107.20)[2]
	EPA	N.E.	59.23 (54.63 - 64.29)[3]
Earthworm (LC_{50})	EPA	N.E.	35.00 (33.43 - 36.64)[3]
	BIOQUEST	N.R.[5]	26.03 (20.15 - 33.84)[4]

[1] No effect.
[2] 95% Confidence Interval in brackets, corrected for variation in stock solution concentration.
[3] Average of 3 toxicity tests.
[4] Average of 4 toxicity tests.
[5] No result.

the initial Daphnia results of BIOQUEST, EP(Edm) and EPA indicated variations that, following two conference calls, were resolved by adjusting the test method to a common water hardness of 200 mg/L for culturing and test control waters. Considerable discrepancy was observed between the BETA and AEC 2-chloroacetamide seed germination test results and the results provided by EPA. Samples of the original stock solutions used by BETA and AEC were sent to EPA for analysis and it was found that some of the discrepency could be explained by lower than anticipated concentrations of chloroacetamide in the stock solutions. As a result, no modifications to the participating laboratories' test procedures were necessary.

The use of reference toxicants is very valuable in standardizing procedures with many different laboratories and test protocols and helped identify and resolve subtle methodology differences between laboratories prior to testing sample sludges and centrifugates.

4.2 GENERAL METHOD OF STATISTICAL ANALYSIS OF RESULTS

Statistical analyses were performed to address three primary objectives:

(1) to assess the within-laboratory levels of precision and reproducibility of three aquatic and two terrestrial toxicity tests on waste sludge samples;

(2) to determine and comment on among-laboratory reproducibility for each of the toxicity assays; and

(3) to comment, based on the results, on the performance of each of the assays.

4.2.1 Within-Laboratory Reproducibility

Within-laboratory precision and reproducibility was assessed by calculating sample means, standard deviations and coefficients of variation (which expresses the among-replicate standard deviation as a percentage of the mean). As all toxicity concentrations were expressed as percentage concentrations of the sludge or centrifugate samples (determined using inverse-prediction from linear regression relationships of percent effect against percent sample concentration), it was necessary to arcsine (angular) transform all data. Since percentages and proportions are distributed according to a binomial rather than normal distribution, these result in estimated error terms (e.g., variance, standard deviation) that are related functionally to the mean, thereby precluding the use of most parametric statistical analyses (Sokal & Rohlf, 1981). Arcsine transformation overcomes this problem by yielding error terms that are independent of the sample means. For clarity of presentation, sample means and corresponding lower and upper standard deviations have been back-transformed into percentage values, although all statistical hypothesis testing was performed on arcsine transformed data.

The coefficient of variation (CV) was calculated on arcsine transformed data as;

$$CV = (1+1/4n)V$$

where; $V = (s\times100)/m$, s = sample standard deviation, m = sample mean, n = sample size. This relationship is used since V alone is a biased estimator of the coefficient of variation especially when small sample sizes are involved (Sokal & Rohlf, 1981).

At times, further assessment using non-parametric ANOVA was performed when it appeared that some differences were not being detected.

4.2.2 Among-Laboratory Variation

Analysis of among-laboratory variation in toxicity results was performed using the analysis of variance statistical procedure (ANOVA) (Sokal & Rohlf, 1981). The analysis is carried out using two steps: (1) test of the null hypothesis that there is no significant difference in mean level of toxicity response among laboratories (i.e. all labs have similar mean response results) using the F-statistic; (2) if the null hypothesis is rejected, perform Tukey's *a posteriori* multiple comparison test between the sample means to determine where the difference(s) occur. The analysis of variance test consists of a comparison between two estimates of the overall variance (of the complete set of measurements included in the analysis), one estimating the variance of the sample means about a grand mean (i.e. between-company variance in test results), and the other based on the variance of the individual measurements about the sample means (i.e. within-company variance in test results). If the null hypothesis is true, the ratio of these two estimates will approximate unity. In contrast,if each of the company responses are different, the ratio will exceed unity. In practice this ratio is equated to the F-statistic which enables the probability of obtaining such a ratio (or one larger), if the null hypothesis were true, to be determined. Since the F-statistic is based on the ratio of two variance estimates, both the numerator and denominator have their own associated degrees of freedom, the numerator having k-1, and the denominator having n-k; where k=number of treatment groups (i.e, laboratories) and n=total number of replicates in the entire data set. If the calculated F statistic exceeds the critical F value obtained from the F-distribution probability table (i.e., $F_{0.05,\ \text{numerator d.f., denominator d.f.}}$) the null hypothesis of equality in toxicity response is rejected. *A posteriori* comparisons are then performed to determine where the differences exist.

Since the toxicity concentrations were presented as percentages, among-laboratory variation analysis was performed on the arcsine transformed values, as was the within-laboratory evaluation.

In some cases, non-parametric ANOVA procedure was additionally used to clarify observed trends (i.e., to determine whether the lack of significance among sample group means was related to high variation in the data when the means were in fact different or whether there were true differences in the mean toxicity responses).

When only two means had to be compared, either a two- or one-sample t-test was used to test the null hypothesis of equality of means.

All hypothesis testing was performed at the probability level of p=0.05.

4.3 DAPHNIA TOXICITY TEST

4.3.1 Test Procedure

This static acute lethality aquatic toxicity test utilizes young (less than 24-hour-old) freshwater macroinvertebrates (the cladoceran Daphnia magna). The mean lethal concentration (LC_{50}) is calculated at 48 hours using both death or immobilization as the end-point.

The Daphnia toxicity tests were conducted according to the procedures for testing chemicals of the Hazardous Waste Assessment Team, U.S. EPA, Corvallis, Oregon (Greene et al., 1988). The specific procedure is presented in Appendix B, and is briefly described as follows:

The tests were carried out with 10 less-than-24-hour-old Daphnia magna in 50 mL of test solution. The Daphnia are derived from strictly controlled stock cultures. A typical test may include, in addition to the 200 mg/L $CaCO_3$ reconstituted deionized water negative control, a test sample series (v:v percent) of 100, 80, 60, 30, 10, 6, 3, and 1 percent (if no previous range finding was performed). If a test solution hardness is found to be below 80 mg/L $CaCO_3$, the solution hardness must be adjusted to 200 mg/L using a defined salt solution. The toxicity test is conducted under predetermined temperature, culture and photo-period conditions, with three replicates at each concentration for a period of 48 hours. The test is considered unacceptable if more than 10 percent of the control organisms die or are immobilized. Mortality and immobility are used to define acute toxicity. The concentration of test material lethal to 50 percent of the population (LC_{50}) and 95 percent confidence limits are then determined from the raw data using the trimmed Spearman-Karber method (Hamilton et al., 1977).

4.3.2 Results

The observed results for the different laboratories and samples are presented in Table 4.3.

All three of the testing laboratories agreed that Sample B was the least toxic. BIOQUEST and EC (Edm) indicate that site A is the most toxic (lowest LC_{50}), but EPA found site C to be the most toxic. The extremely small LC_{50} for Sample A presented by BIOQUEST is questionable with respect to the ability to accurately and homogeneously dilute the gas plant samples to this low level (and lower, for interpolation purposes).

4.3.3 Statistical Assessment of Daphnia Test Reproducibility

The intra-laboratory variability is represented by the coefficient of variation and is presented in Table 4.4.

For the *Daphnia* toxicity test, it appears that the highest variability for all three laboratories is observed in Sample C.

Inter-laboratory variability was analysed using ANOVA analysis and these results are presented in Table 4.5.

When the mean responses are compared using ANOVA analysis, differences are observed within the sample sites. For Sample A, all companies are significantly different. EPA and EC (Edm) are comparable for Sample B, but Bioquest is significantly different. Sample C shows that Bioquest and EPA are similar but EC (Edm) is significantly different from them.

Table 4.3. Summary statistics for _Daphnia magna_ LC_{50} (% vol/vol) toxicity tests.

	Mean LC_{50}		
	Sample A	Sample B	Sample C
BIOQUEST	LT[1] 0.1 (NA[2])	17.7 (16.2-19.3)[3]	0.5 (0.4-0.6)
EPA	2.3 (2.2-2.5)	36.1 (34.3-37.8)	0.2 (0.1-0.2)
EC (Edm)	0.7 (0.7-0.7)	34.6 (31.8-37.4)	13.5 (10.4-16.9)

Note: Results have been rounded up to one decimal point.

[1] Less than.
[2] Not applicable.
[3] 95% confidence interval in brackets.

Table 4.4. Coefficients of variation, as percentages, for Daphnia magna LC_{50} toxicity tests.

Laboratory	Coefficient of Variation (%) Sample A	 Sample B	 Sample C
BIOQUEST	6.7	5.1	14.1
EPA	3.7	3.2	7.5
EC (Edm)	2.1	5.1	13.7

Table 4.5 Summary of ANOVA analysis in Daphnia magna LC_{50} estimates.

Sample	Laboratory			ANOVA Critical F
A	BIOQUEST (LT[1] 0.1)[2]	EPA (2.3)	EC (Edm) (0.7)	$F_{2,6}$ = 1619.5 **
B	BIOQUEST (17.7)	EPA (36.1)	EC (Edm) (34.6)	$F_{2,5}$ = 64.6 **
C	BIOQUEST (0.5)	EPA (0.2)	EC (Edm) (13.5)	$F_{2,6}$ = 133.0 **

[1] Less than.

[2] Mean Values in brackets.

NS Not significant : P is greater than or equal to 0.05.

* P is less than 0.05 : significant.

** P is less than 0.01 : highly significant.

NOTE: Group means are given in parentheses. Means with a common suprascore are not significantly different as determined using Tukey's Honest Significant Difference "a posteriori" test.

Results indicate that all laboratories possess good reproducibility, as represented by coefficients of variation (CV) of less than 20 percent. Variations in the CV, however, are observed with the samples as well as with laboratories. Based on the ANOVA results for Sample A, all laboratories vary from each other. This may be the result of small values for the means, combined with low variation in the tests, leading to statistically separate laboratories. Statistical analysis of variance for Samples B and C supports the readily observed fact that, for each sample, one of the laboratories produced results not comparable with the other two laboratories.

4.4 ALGAL TOXICITY TEST

4.4.1 Test Procedure

This static acute sublethal aquatic toxicity test utilizes the freshwater algae, Selenastrum capricornutum, and is conducted in 96 hours. After this time, the lowest concentration causing inhibition of growth by 50 percent relative to the control is determined (EC_{50}), along with 95 percent confidence intervals using the trimmed Spearman-Karber method (Hamilton et al., 1977).

The algal toxicity tests (bottle method) were conducted according to the procedures of the Hazardous Waste Assessment Team, U.S. EPA, Corvallis, Oregon (Porcella, 1983) and a microplate method was conducted according to the methodology of Blaise et al. (1986). The specific procedure is presented in Appendix B and is briefly described as follows:

Stock cultures of Selenastrum capricornutum (a unicellular, non-motile chlorophyte) are kept under controlled growth conditions in a defined algal assay medium (AAM). Full strength AAM is used to dilute site samples for the test sample series. Fifty millilitres of test solution are dispensed into each of three replicate 125 mL flasks to obtain an eluate test series of logarithmic concentrations from 0 to 80 percent, in addition to an AAM negative control. A 4 to 5-day stock culture is used as the inoculum source, whose population density was determined by electronic or direct counting. An inoculum of algae from the stock solution provides a test solution concentration of 10 000 cells/mL at the start of the test (the pH must be between 6 and 10). Incubation under controlled light and temperature conditions occurs with continuous shaking for 96 hours. Algal growth is then measured and the lowest concentration inhibiting growth by 50 percent (EC_{50}) is calculated using linear regression analysis.

A modification of this procedure based on microassay techniques (Blaise, et al, 1986) was used by the ENVIRONMENT CANADA (Edmonton) laboratory and the specifics of this procedure are presented in Appendix B.

Sample A was observed to have an oil phase, which was removed prior to toxicity testing but may have contributed to the observed variability.

4.4.2 Results

The observed results are presented in Table 4.6.

Comparison of observed sample toxicities agree with the trend observed with the Daphnia test results, i.e., Site A was more toxic than Site B. When the 95% confidence interval is considered, the mean toxicity in Sites B and C appear similar.

4.4.3 Statistical Assessment of Algal Test Reproducibility

The variability within each laboratory, on a per sample basis, is presented in Table 4.7.

The coefficient of variation could not be calculated for EPA since their results were presented as a mean of the three replicates and could not be separated into individual replicates without complete re-evaluation of the raw data. EC (Edm) shows consistent reproducibility for all three site samples, with coefficients of variation between 16 and 22 percent.

A one-sample t-test was used to compare the samples from EPA and EC (Edm) and these results are presented in Table 4.8.

Statistical comparison between laboratories was performed

Table 4.6. Summary statistics for Selenastrum capricornutum EC_{50} (% vol/vol) toxicity tests.

Laboratory	Mean EC_{50}		
	Sample A	Sample B	Sample C
EPA	0.2 (0.1-0.3)[1]	25.2 (20.0-45.0)	12.3 (7.5-16.5)
EC (Edm)	0.8 (0.6-1.1)	29.5 (19.9-40.1)	22.4 (15.5-30.1)

Note: Results have been rounded up to one decimal point.

[1] 95% confidence interval in brackets.

Table 4.7. Coefficients of variation for Algae (Selenastrum capricornatum) EC_{50} toxicity tests.

	Coefficient of Variation (%)		
Laboratory	Sample A	Sample B	Sample C
EPA	-[1]	-	-
EC (Edm)	16.8	21.1	19.3

[1] Not readily available from results provided.

Table 4.8. Summary of one-sample t-test for Algae toxicity tests.

Sample	Laboratory		Critical t_{df}[1]
A	EPA (0.2)[2]	EC (Edm) (0.8)	t_2 = 5.58 *
B	EPA (25.2)	EC (Edm) (29.5)	t_2 = 2.74 NS
C	EPA (12.3)	EC (Edm) (22.4)	t_2 = 2.66 NS

NS Not significant : P is greater than or equal to 0.05.
* P is less than 0.05 : significant.
[1] d.f degrees of freedom.
[2] Mean values in brackets.

NOTE: Group means are given in parentheses. Means with a common suprascore are not significantly different as determined using Tukey's Honest Significant Difference "a posteriori" test.

even though EC (Edm) utilized the microassay modification of the method and EPA performed the bottle-flask method.

No statistically significant difference is observed between the laboratories for Sample B or C. This, combined with the overlap of the 95% confidence intervals for EC (Edm) samples B and C, suggests similar toxicity behaviour of these two samples. A significant difference is observed between EPA and EC (Edm) laboratories for Site A.

It is interesting to note that, even though two variations of the technique were used by the laboratories, there is no significant variation between each other for Samples B and C. A statistically significant difference was observed with Sample A, the most toxic in this test. This is similar to the results observed with Daphnia where low variation, associated with the small sample mean value, produces a statistical separation of the two sets of results. Although statistically different, it is intuitively apparent that absolute EC_{50} values of less than one percent would be considered biologically the same. This result demonstrates the two different goals of result comparison, one being relative biological effect and the other being laboratory reproducibility of results.

4.5 MICROTOX TOXICITY TEST

4.5.1 Test Procedure

This static acute aquatic toxicity test utilizes a marine bioluminescent bacterium, Photobacterium phosphoreum, and is conducted in a very standardized fashion following the protocol of the supplier, Microbics Ltd. and the method used by the Alberta Environmental Center, Vegreville. The samples are analyzed and EC_{50} and EC_{20} values are calculated according to standardized methods, for both a 5-minute and 15-minute duration.

The standard procedure is generalized as follows:

One millilitre of Microtox reconstitution solution is placed in a cuvette and kept at 4°C. After 15 minutes, a vial of Microtox reagent at 4°C is opened and the reconstitution solution added, swirled and transferred back into the cuvette and kept at 4°C.

Analyses are conducted using Microtox reagents and cuvettes, with light measurement performed by a photometer with a reaction chamber at 15°C. Prior to analysis, each sample is adjusted to contain 2.0 percent sodium chloride by addition of a 22 percent NaCl solution. A dilution series for the samples is then prepared in 2 percent NaCl in glass cuvettes and maintained at 15°C. The Microtox bacteria are prepared by dispensing 500 µL of Microtox diluent into 9 cuvettes, adding 10 µL Microtox bacteria stock suspension, mixing and maintaining at 4°C for 15 minutes. The light emitted from each cuvette is then measured and, at timed intervals, 500 µL from each sample dilution is added to a cuvette and mixed. This is performed in duplicate. The light emitted from the bacteria is measured after 5 and 15-minute exposures to the sample. A standardized procedure is then used to determine the EC_{20} and EC_{50} values. A more detailed discussion of the method is presented in Appendix B.

Multiple phases were observed in Samples A and C. This potential problem had previously been identified and the recommended method of dealing with it was to separate phases and analyze the aqueous phase. All laboratories were to follow this procedure if any laboratory experienced multiple phases with a sample. ERCB, ESSO and BETA did the separation but it is unknown if EC (Edm) did.

Precipitate was also noted in Sample B by BETA and ERCB.

4.5.2 Results

The observed Microtox EC_{20} results are presented in Table 4.9 and the EC_{50} results are shown in Table 4.10.

The EC_{20} results, for 5 minutes and 15 minutes, both present the same pattern for ranking the samples. ERCB and ESSO specify Sample A to be the most toxic and Sample B to be the least toxic, which is generally consistent with the daphnid and algal toxicity tests. BETA, however, evaluated Sample C to be similar to Sample B. It should be noted that for both EC_{20} test durations, BETA obtained 95% confidence intervals for samples B and C that overlapped extensively.

Table 4.9. Summary statistics for Microtox EC_{20} (% vol/vol) toxicity tests.

Laboratory	Mean EC_{20}		
	Sample A	Sample B	Sample C
5 Minute			
BETA	0.3 (0.1-0.6)[1]	4.7 (3.6-5.9)	5.1 (4.0-6.2)
ERCB	0.9 (0.6-1.2)	9.4 (7.7-11.4)	2.1 (1.1-3.5)
ESSO	0.5 (NA)[2]	26.3 (10.3-46.6)	0.9 (LT0.1[3]-4.1)
15 Minute			
BETA	0.2 (0.1-0.5)	4.3 (3.0-5.7)	4.9 (4.0-5.9)
ERCB	0.3 (0.2-0.4)	6.8 (5.7-7.9)	2.0 (0.8-3.9)
ESSO	0.3 (0.1-0.6)	19.0 (7.5-34.3)	1.0 (LT0.1-4.2)

Note: Results have been rounded up to one decimal point.

[1] 95% confidence interval.
[2] Not available.
[3] Less than.

Table 4.10. Summary statistics for Microtox EC_{50} (% vol/vol) toxicity tests

Laboratory	Mean EC_{50}		
	Sample A	Sample B	Sample C
5 Minute			
BETA	1.0 (0.3-1.9)[1]	23.3 (20.2-26.4)	22.4 (21.8-23.0)
EC (Edm)	0.2 (0.2-0.2)	18.2 (17.7-18.8)	17.4 (14.4-20.5)
ERCB	2.0 (1.5-2.6)	43.1 (37.6-48.7)	15.2 (9.0-22.8)
ESSO	1.4 (1.3-1.5)	75.8 (37.1-98.7)	5.1 (LT[2]0.1-18.1)
15 Minute			
BETA	0.9 (0.3-1.7)	25.3 (21.1-29.7)	24.5 (23.7-25.3)
EC (Edm)	0.2 (0.2-0.2)	20.7 (20.6-20.7)	18.8 (16.7-21.1)
ERCB	0.7 (0.5-0.9)	42.4 (34.0-51.1)	15.9 (8.2-25.6)
ESSO	0.8 (0.5-1.2)	NA[3]	4.5 (LT0.1-18.5)

Note: Results have been rounded up to one decimal point.

[1] 95% confidence interval.
[2] Less than.
[3] Not available.

Comparison of the 5-minute to the 15-minute EC_{20} values shows a minimal decrease in the EC_{20} value, i.e. increased toxicity. An exception to this is observed with ESSO for Sample C. It should be noted that ESSO obtained an EC_{20} value of 0.0 percent for one of the replicates during each of the Sample C EC_{20} time durations.

All participating laboratories consistently produced EC_{50} values, both at 5 minutes and 15 minutes, presenting the lowest value with Sample A (most toxic) and the highest value with Sample B (least toxic) (Table 4.10). Extensive overlap of the 95% confidence intervals was observed for Samples B and C in the BETA and EC (Edm) results. It should be noted that ESSO presented two of the three replicates for Sample B at the 15-minute test duration with values in excess of 100 percent. These results were a consequence of extrapolation of their data.

Comparison of the 5-minute to the 15-minute EC_{50} test results shows more variability in observed trends than the EC_{20} test. A trend of increasing toxicity (decreasing EC_{50} value) is observed among the laboratories only for Sample A. EC (Edm) presents equal or greater EC_{50} values for the 15-minute test than for the 5-minute test for all samples tested, while BETA presents greater EC_{50} (15-minute) values for Samples B and C. The ERCB presents a slightly greater (but similar confidence intervals) value for Sample C EC_{50} (15 min) only and ESSO presents lower EC_{50} (15 min) than EC_{50} (5 min) values for all samples.

4.5.3 Statistical Assessment of Microtox Test Reproducibility

The coefficients of variation for the EC_{20} test are presented in Table 4.11 and those for the EC_{50} test are presented in Table 4.12.

An overview of the CV values for EC_{20} values of both 5 minute and 15-minute duration shows that ESSO presents a CV value over 100 percent for Sample C. It can be seen in Appendix C that a value

Table 4.11. Coefficients of variation (as percentages)for Microtox EC_{20} toxicity tests.

Laboratory	Coefficient of Variation (%) Sample A	Sample B	Sample C
EC_{20} (5 min.):			
BETA	49.0	13.1	12.0
ERCB	19.7	10.9	31.6
ESSO	NA[1]	42.7	116.7
EC_{20} (15 min.):			
BETA	49.3	17.4	10.7
ERCB	19.3	8.7	41.7
ESSO	40.8	41.7	111.9

[1] Not available.

Table 4.12. Coefficients of variation (as percentages) for Microtox EC_{50} toxicity tests.

	Coefficient of Variation (%)		
Laboratory	Sample A	Sample B	Sample C
EC_{50} (5 min.):			
BETA	43.0	7.8	1.6
EC (Edm)	5.4	1.8	10.2
ERCB	13.8	8.5	26.2
ESSO	3.9	41.2	100.2
EC_{50} (15 min.):			
BETA	40.9	10.2	1.9
EC (Edm)	5.4	0.2	6.8
ERCB	15.0	13.2	33.8
ESSO	21.2	NA[1]	115.6

[1] Not available.

of zero was recorded for one of their results. If ESSO's Sample C is not considered, the reproducibility within the participating laboratories is generally good. It can be noted, however, that the variability within a laboratory is not as consistent through samples as for the previous toxicity tests.

The within laboratories results for the EC_{50} tests generally present somewhat less variability than for the EC_{20} tests. Discounting ESSO's CV in excess of 100 percent for Sample C, the only other notably significant CV values are for BETA's Sample A and ESSO's Sample B. Both of these may be the result of modifications from the standardized protocol, such as extrapolation of data to obtain the results. The comparison between laboratories for the EC_{20} test using ANOVA analysis is presented in Table 4.13.

For the EC_{20} test results, ANOVA analysis indicates that all laboratories are statistically comparable except for Sample B at 5-minutes duration. These results show that the ERCB and ESSO are similar but BETA and ESSO are significantly different.

The sample toxicity ranking observed in the results appears to be overshadowed by some relatively large variations within laboratories, as can be seen in the tables presenting the coefficients of variation. This results in no statistically significant variation with most of the means.

When a supplemental non-parametric ANOVA analysis was performed on the results for the 15-minute EC_{20} test on Sample B (where the EC values of greater than 100 percent were observed for ESSO), a significant difference is now observed between ESSO and BETA/ERCB.

The comparison between laboratories for the EC_{50} tests is presented in Table 4.14.

Table 4.13. Summary of ANOVA analysis for Microtox EC_{20} toxicity tests.

Sample	Laboratory			Critical F
EC_{20} (5 min.):				
A	BETA (0.3)	ERCB (0.9)	ESSO (0.5)	$F_{2,6}$ = 3.88 NS
B	BETA (4.7)	ERCB (9.4)	ESSO (26.3)	$F_{2,6}$ = 5.22 *
C	BETA (5.1)	ERCB (2.1)	ESSO (0.9)	$F_{2,6}$ = 2.84 NS
EC_{20} (15 min.):				
A	BETA (0.2)	ERCB (0.3)	ESSO (0.3)	$F_{2,6}$ = 0.10 NS
B	BETA (4.3)	ERCB (6.8)	ESSO (19.0)	$F_{2,6}$ = 4.61 NS
C	BETA (4.9)	ERCB (2.0)	ESSO (1.0)	$F_{2,6}$ = 2.36 NS

NS Not significant : P is greater than or equal to 0.05.
* P is less than 0.05 : significant.
** P is less than 0.01 : highly significant.

NOTE: Group means are given in parentheses. Means with a common suprascore are not significantly different as determined using Tukey's Honest Significant Difference "a posteriori" test.

Table 4.14. Summary of ANOVA analysis for Microtox EC_{50} toxicity tests.

Sample	Laboratory				Critical F
EC_{50} (5 min.):					
A	EC (Edm) (0.2)	BETA (1.0)	ERCB (2.0)	ESSO (1.4)	$F_{3,8}$ = 11.02 **
B	EC (Edm) (18.2)	BETA (23.3)	ERCB (43.1)	ESSO (75.8)	$F_{3,8}$ = 5.58 *
C	EC (Edm) (17.4)	BETA (22.5)	ERCB (15.2)	ESSO (5.1)	$F_{3,8}$ = 2.78 NS
EC_{50} (15 min.):					
A	EC (Edm) (0.2)	BETA (0.9)	ERCB (0.7)	ESSO (0.8)	$F_{3,8}$ = 3.43 NS
B	EC (Edm) (20.7)	BETA (25.3)	ERCB (42.4)	ESSO NA	$F_{3,8}$ = 4.46 *
C	EC (Edm) (18.8)	BETA (24.4)	ERCB (15.9)	ESSO (4.5)	$F_{3,8}$ = 2.98 NS

NS Not significant : P is greater than or equal to 0.05.
* P is less than 0.05 : significant.
** P is less than 0.01 : highly significant.
NA Not available.

NOTE: Group means are given in parentheses. Means with a common suprascore are not significantly different as determined using Tukey's Honest Significant Difference "a posteriori" test.

Although the laboratories behave unpredictably with respect to their EC_{50} inter-laboratory variability, a few generalizations can be made. Firstly, all laboratories' means are statistically comparable for both EC_{50} test durations for Sample C, based on ANOVA results. Additionally, EC (Edm) and ESSO generally are on opposing extremes of the observed EC_{50} mean values and variations, with EC (Edm) on the low side and ESSO on the high side. ESSO, as previously mentioned, presented EC_{50} (15 min) values for sample B exceeding 100 percent, as well as showing wide ranges of values.

An additional non-parametric analysis of Sample C for the 5-minute EC_{50} test resulted in significant differences between ESSO and the other participants.

It appears that proper adherence to the recommended technique and analysis protocol was not followed by all the laboratories involved in Microtox testing. In the future, the test protocol should be more detailed, if within-laboratory and among-laboratory reproducibilities are to be optimized. This is made evident when statistical evaluations test these laboratory data reproducibilities. Another possibility for significant differences in results may be due to unavoidable sample non-homogeneity.

When one excludes the cases where these protocols do not appear to have been followed, good reproducibility of results is generally observed. It is noted that the 15-minute test duration appears to have somewhat less variability than the 5-minute test. The EC_{20} test also appears to generate a greater extent of statistical agreement (based on ANOVA) than does the EC_{50} test, which may be the result of more variation in the EC_{20} results as indicated by the larger CV's.

The statistical analysis produces an indicator of agreement between participants and determines the degree of confidence that can be placed on the accuracy of the results. ANOVA analysis generates a parameter that is produced by a combined effect of the magnitude of the mean and the extent of variation. When the variation is large relative to the estimated mean, no statistical difference can be detected among participants. However, when the parametric ANOVA is supplemented with a non-parametric analysis of variance, these outliers may often be identified, as was seen with ESSO for the Sample C EC_{50} (5 min) and for ESSO's Sample B EC_{20} (15 min) test results.

The statistically-derived within-sample significant differences that indicate potential problems with data reproducibility, however, do not interpret directly into biological significance. Laboratories may report statistically different toxicities for a sample but biologically the toxicity may be the same. Conversely, some results may be statistically similar to other results based on their large variability, but comparison of the means and confidence intervals indicates there may be different biological impacts. The interpretation of the results should be dictated by the specific concern applicable to any given sample analysis, i.e. reproducibility of results by laboratories or sample toxicity to a biological system. This study focussed on reproducibility concerns.

4.6 SEED GERMINATION TOXICITY TEST

4.6.1 Test Procedure

This static terrestrial toxicity test assesses the inhibition of seed germination to indicate toxicity of the material being tested. Toxic effects due to herbicides, heavy metals, salinity, toxic chemicals or disposal of waste on land can easily be detected with this method. The test has a duration of 120 hours, after which the results are analyzed to determine an EC_{50} value. The same chemical may induce different responses in different plant species. In this study, it was decided to utilize cucumber (hybrid Calypso) (Cucumis sativa) and barley (Hordeum vulgare) as representative species of economic importance. The critical end-point of the test is seed germination.

The seed germination soil toxicity tests were conducted according to the procedures for testing chemicals of the Hazardous

Waste Assessment Team, U.S. EPA, Corvallis Environmental Research Laboratory (Greene et al., 1988). The detailed procedure is presented in Appendix B and is briefly discussed below.

The test utilizes untreated seeds which must be size-graded and the most prevalent size class is used for testing. The EC_{50} estimation requires three replicates of a negative control and at least five test soil concentrations chosen in a geometric series, such as 80, 40, 20, 10, and 5 percent (w:w) site soil or sediment. Each petri dish, containing 100 grams of air-dried soil, is seeded with 40 seeds, and evenly covered with 90 grams of sand and irrigated with distilled water to 85 percent of its water holding capacity. Incubation is under controlled temperature and light conditions for a period of 120 hours, after which percent germination is determined. At least 80 percent of the seeds in the control dishes must germinate. The EC_{50} value is then calculated using the trimmed Spearman-Karber technique (Hamilton, 1977).

4.6.2 Results

The results from the different participating laboratories are presented in Table 4.15.

The general trend observed with both barley and cucumber has Sample A as the most toxic and Sample B as the least toxic, in agreement with the general pattern observed in the other toxicity tests. Exceptions, which rank Sample C, rather than B, as the least toxic, can be seen with BETA for both cucumber and barley, and with EPA for cucumber.

Comparison of the barley and cucumber results also present a generally lower EC_{50} value for barley than for cucumber.

4.6.3 Statistical Assessment of Seed Germination Test Reproducibility

Presentation of the variation of results for both cucumber and barley are presented in Table 4.16.

The CV results show that there are fluctuations in CV values both within laboratories and within samples.

Table 4.15. Results summary for seed germination toxicity tests.

Laboratory	Mean EC_{50} [1]		
	Sample A	Sample B	Sample C
Cucumber			
AEC	8.5 (7.9-9.2)[2]	36.7 (31.2-42.4)	20.9 (12.1-31.5)
BETA	2.1 (1.0-3.7)	30.1 (25.4-35.0)	37.4 NA[3]
EPA	6.8 (6.4-7.3)	18.3 (13.7-23.4)	7.8 (6.1-9.7)
Barley			
AEC	4.6 (3.6-5.7)	28.6 (24.6-32.7)	19.5 (15.6-23.7)
BETA	LT[4]1.0 NA	3.5 (3.3-3.7)	36.0 (34.9-37.1)
EPA	3.8 (3.6-4.1)	19.0 (16.7-21.3)	22.3 (15.2-30.4)

---

[1] Based on percent vol/vol.

[2] 95% confidence interval.

[3] Not available since only one data point (other two replicate data resulted in error generation using the Trimmed Spearman-Karber method).

[4] Less than.

Table 4.16. Coefficients of variation, as a percentage, for seed germination toxicity tests.

Laboratory	Coefficient of Variation (%) Sample A	Sample B	Sample C
Cucumber			
AEC	4.5	9.8	27.3
BETA	34.6	9.8	–[1]
EPA	4.0	15.4	12.7
Barley			
AEC	12.5	8.7	12.1
BETA	–[2]	2.7	2.0
EPA	3.6	7.1	20.2

[1] Single Estimate.
[2] Below Detection Limits.

It can also be generally stated that CV for barley are smaller than those for cucumber, suggesting somewhat improved experimental reproducibility.

All coefficients of variation are at an acceptable level of less than 35, with most presenting good laboratory reproducibility with CV's of less than 15.

The results of the analysis of variance for the seed germination tests are summarized in Table 4.17.

For the sample with the lowest EC_{50} value, Sample A, AEC and EPA show no significant differences for either the barley or cucumber tests. Significant difference is observed between the AEC results and BETA results for both tests. The significant variation shown in the barley analysis of Sample B appears to be a problem with accuracy, i.e. high variability in the reported mean toxicity value.

The seed germination test, utilizing cucumber and barley, appears to present the most variable results of all the toxicity tests performed. The general reproducibility within laboratories is at acceptable levels but the variability arises from the magnitudes of the derived means. This would suggest that further procedural improvements may be necessary to improve the reproducibility among laboratories.

It should also be noted that the test using barley appears more sensitive than the test using cucumber.

4.7 EARTHWORM TOXICITY TEST

4.7.1 Test Procedure

The second static acute terrestrial toxicity test employs earthworms (Eisenia andrei) as an indicator of the toxicity of the test samples. In contrast to aquatic organisms, earthworms may be exposed to toxic chemicals through various pathways, such as via the aqueous phase, the vapor phase and by direct contact (absorption, ingestion). Soils also have the ability to immobilize chemicals, especially organics, by adsorption.

Table 4.17. Summary of ANOVA analysis for toxicity tests for seed germination.

Sample	Laboratory			Critical F
Cucumber				
A	AEC (8.5)[1]	EPA (6.8)	BETA (2.1)	$F_{2,6}$ = 23.19 **
B	AEC (36.7)	EPA (30.1)	BETA (18.3)	$F_{2,6}$ = 10.04 *
C	AEC (37.4)[2]	EPA (20.9)	BETA (7.8)	$F_{2,6}$ = 7.87 *
Barley				
A	AEC (4.6)	EPA (3.8)		$F_{1,4}$ = 1.88 NS
B	AEC (28.6)	EPA (19.0)	BETA (3.5)	$F_{2,6}$ = 113.79 **
C	AEC (19.5)	EPA (22.3)	BETA (36.0)	$F_{2,6}$ = 7.94 *

NS Not significant : P is greater than or equal to 0.05.
* P is less than 0.05 : significant.
** P is less than 0.01 : highly significant.

[1] Mean given in brackets.

[2] Single estimate.

The earthworm bioassays were conducted according to the procedures for testing chemicals of the Hazardous Waste Assessment Team, U.S. EPA, Corvallis Environmental Research Laboratory (Greene et al., 1988). Specifics of the procedure are presented in Appendix B. The test organism stock cultures must be properly keyed out to verify the correct species. Tests require adults (at least 2 months old with a clitellum) weighing from 300 to 500 mg and being derived from the same culture. Worm stock cultures are grown in peat moss bedding, pH adjusted between 5 and 8 by adding up to 3 percent by weight of $CaCO_3$. Crowding is to be avoided. Water is added to keep the culture moist but not to allow collection of water in the container.

A test soil is prepared by mixing homogenized waste with artificial soil (10% sphagnum peat, 20% kaolinite clay, 70% sand) to prepare geometric test soil concentrations, such as 80, 40, 20, 10, 5 and 0 percent dry weight/dry weight. The final artificial soil pH is adjusted to 6.5 ± 0.5 with pulverized calcium carbonate. Site soil pH must be within the range of 5 to 9, otherwise it must be adjusted with either 5.0 N NaOH or 5.0 N HCl to achieve this criterion. Test soils are based on the ratio of site soil to artificial soil. Once mixed, the test soils are hydrated with distilled or deionized water, based on the water holding capacity, so as to create a moist environment (75 percent of water holding capacity).

The testing itself consists of placing ten healthy, purged earthworms into each of three replicate jars containing 200 grams (dry weight) of test soil. The worms are not fed during the test. The jars are kept at 20 ± 2°C under continuous light, and mortality is assessed at 7 and 14 days. The results are generated by treating the data with the trimmed Spearman-Karber method (Hamilton et al., 1977) to generate LC_{50} values.

4.7.2 Results

The LC_{50} results for the participating laboratories are presented in Table 4.18.

Problems were encountered with the wetness of the sludge samples and the resulting saturation of the test soil. This is a possible explanation for the lack of test endpoint quantification i.e. greater than 10.0, of the Sample C LC_{50} for BIOQUEST. Both EPA and BIOQUEST Sample A results indicated that the sludge/soil mixtures were extremely toxic at concentrations as low as 0.6%. Definite results could not be obtained for Sample A since the toxicity could not be reliably measured below 0.6% v/v sludge in soil.

For the results presented, the toxicity appears highest for Sample A and lowest for Sample C.

4.7.3 Statistical Assessment of Earthworm Test Reproducibility

Presentation of the variation within laboratory results is summarized in Table 4.19.

The available coefficients of variation are all quite acceptable, with EPA exhibiting a lower level than BIOQUEST. For Sample B, greater variation is observed with BIOQUEST than with EPA but, again, they are all within good levels of reproducibility.

Comparison between laboratories was performed when possible and the results are presented in Table 4.20.

The only analysis of variance that could be performed, due to a lack of adequate data, was for Sample B. It was observed that there was no statistically significant variation between the laboratories.

As a consequence of the limited results, statistical evaluation was limited to Sample B, where no statistically significant variation between laboratories was observed. Variation within laboratories was also at low levels, as indicated by the low CV values.

Problems with the procedure, such as how to deal with the wetness of the sludge, are being researched at EPA, Corvallis (J. Greene, personal communication). Resolution of these problems will permit generation of complete data sets, from which more meaningful statistical evaluations may arise.

Table 4.18. Summary statistics for *Eisenia* LC_{50} (% vol/vol) toxicity tests.

Laboratory	Mean LC_{50}		
	Sample A	Sample B	Sample C
BIOQUEST	LT[1]0.6 NA[3]	3.0 (2.3-3.8)[4]	GT[2]10.0 NA
EPA	LT0.6 NA	3.2 (2.9-3.5)	25.7 (24.7-26.6)

[1] Less than.
[2] Greater than.
[3] Not applicable.
[4] 95% confidence interval.

Table 4.19. Coefficients of variation (as percentages) for *Eisenia andrei* toxicity tests.

Laboratory	Coefficient of Variation (%) Sample A	 Sample B	 Sample C
BIOQUEST	NA[1]	14.0	NA
EPA	NA	5.5	2.2

[1] Not applicable.

Table 4.20. Summary of t-test analysis for toxicity tests for *Eisenia andrei* EC_{50}.

Sample	Laboratory		Critical t_{df}
A	BIOQUEST (LT 0.6)[1]	EPA (LT 0.6)	-
B	BIOQUEST (3.0)	EPA (3.2)	$t_{(4)} = 0.49$ NS
C	BIOQUEST (GT 10.0)	EPA (25.7)	-

NS not significant : P is greater than 0.05.

[1] Mean value given in brackets.

4.8 S.O.S. CHROMOTEST

In contrast to acute toxicity tests, the S.O.S. Chromotest can be used to detect genotoxicity. This test was performed by EPA and EC (Long.) with the intent of providing some additional information on the gas plant sludge genotoxicity potential.

4.8.1 Test Procedure

The test organism is a bacterial mutant of *Escherichia coli* that has been genetically engineered. A normally absent gene for the enzyme beta-galactosidase has been linked to a DNA repair system (S.O.S.) gene operator. This permits colorimetric detection of beta-galactosidase which is consequently produced when the S.O.S. repair mechanism is activated. Activation of this DNA repair system is an indicator of induced damage to the DNA, suggesting genotoxicity of the chemical being tested. To detect genotoxicity of chemicals requiring metabolic conversion prior to causing DNA damage, a rat liver extract (S9) can be added to the bacterial-chemical suspension. Specific descriptions of the procedures involved are obtained from Clonetics Corporation (1986).

This test, as well as other mutagenic and genotoxic tests, are currently in the developmental phase and will require further study to determine their applicability to hazardous or toxic material characterizations.

The S.O.S.-inducing potency is the test indicator of genotoxicity and represents the induction factor per nanomole of compound tested, taken from the linear region of the dose-response curve. The induction factor is the ratio of the enzyme activities, beta galactosidase/alkaline phosphatase at concentration C of the site tested, divided by its value at concentration 0.

4.8.2 Results

The centrifugate sample results obtained by EPA and EC (Long) indicated that none of the interstitial liquids were genotoxic.

If a decision is made to pursue genotoxic testing, it is recommended that comparisons be performed against acute toxicity tests using a variety of sludge liquid types, as well as comparison with other genotoxicity/mutagenicity screening tests such as Ames test. This could provide information on the various test sensitivities to various types of sludge fluids.

4.9 TOXI-CHROMOTEST

The bacterial Toxi-Chromotest was performed by EPA, following the procedure specified by Clonetics (1986).

4.9.1 Test Procedure

The Toxi-Chromotest utilizes a freshwater, highly permeable mutant of *Escherichia coli* and a colorimetric assay for the activity of an induced enzyme similar to the assay used for the S.O.S. Chromotest. In the Toxi-Chromotest, the bacteria are stressed by exposure to potential toxicants. Following this, they are placed in a recovery medium with beta-galactosidase inducer and substrate. If the material that the bacteria were exposed to was toxic, cell activity would be affected so that the colorimetric indicator of beta-galactosidase activity would be negative, i.e. no color produced. If the cell was not damaged, enzyme activity would produce a colorimetric response. This test differs from the S.O.S. Chromotest in the location of the enzyme-producing system and therefore these tests respond to different cell stresses.

4.9.2 Results and Comparison to Microtox Response

The results obtained by EPA are presented in Table 4.21. Site A was observed to exhibit a response, while the other sites showed no effect.

When compared to the Microtox results of this study (see Section 4.5.2), it appears that the Toxi-Chromotest is less sensitive to the materials available in the sites sampled, as no effect was most often observed. The maximum testable concentration for the

Table 4.21. Toxi-Chromotest bacterial assay results.

Parameter	EC_{50} (% v/v)		
	Site A	Site B	Site C
Mean	15.4 (11.3-21.5)[2]	NE[1]	NE

[1] NE No effect.

[2] 95% confidence limit.

Toxi-Chromotest is 33.3 percent due to its experimental design (maximum testable concentration for Microtox is 50% v/v). This limits the range of samples which can be tested. This is seen in the Sites B and C samples showing no effect while the Microtox test was capable of establishing a toxic level.

4.10 EFFECT OF pH ADJUSTMENT

Extreme pH levels of liquid centrifugate and/or leachates from two of the four sludge samples evaluated during the Phase II study had a significant effect on the degree of acute toxicity observed. Some of the liquid samples in that study possessed a pH range of 3.2 to 11, leading to the recommendation that pH adjustment of liquid fractions be undertaken, with the realization that the non-pH induced toxicity may be altered by the neutralization process. If, however, one wishes to determine the degree of toxicity due to excess hydrogen ions, the toxicity of pH adjusted and unadjusted split samples should be conducted and the results compared.

During this study, sample pH was between 6 and 9, resulting in the decision that the samples would not be pH-adjusted. However, a duplicate run on the Site A sample was evaluated by EC (Edm) on the Microtox and algae toxicity tests. The unadjusted sample pH was 8.24 and the adjusted pH was 7.04. These results are presented in Table 4.22.

Comparison of the tabulated values indicates that, for Microtox, the toxic limit (EC_{50}) has been minimally raised by the pH adjustment. The algal test results (95 percent confidence intervals only) suggest that the pH adjustment may have resulted in a reverse effect to the toxicity. However, to fully determine the effect of pH on toxicity results, more detailed experimental design and statistical analysis would need to be performed.

This brief study on pH was unfortunately performed on the most toxic sample (lowest EC_{50}). As is shown in the toxicity test results, the small EC_{50} values make it more difficult to clearly intrepret the responses.

Table 4.22. pH adjustment effect on Microtox and Algae results.

Sample	Replicate	EC_{50} (% v/v)		
		Microtox 5-min	Microtox 15-min	Algae 96 hr.
Site A, pH = 8.24	1	0.2 (0.1-0.3)[1]	0.2 (0.2-0.2)	0.8 (0.4-1.3)
	2	0.2 (0.2-0.3)	0.2 (0.2-0.3)	1.1 (1.0-1.3)
	3	0.2 (0.2-0.3)	0.2 (0.2-0.3)	0.6 (0.1-1.1)
Site A, pH = 7.04	1	0.3 (0.2-0.5)	0.3 (0.2-0.6)	-[2] (0.3-0.6)
	2	0.3 (0.3-0.4)	0.4 (0.3-0.5)	- (0.3-0.6)
	3	0.3 (0.2-0.3)	0.3 (0.2-0.3)	- (0.3-0.6)

NOTE: Results have been rounded up to one decimal point.

[1] Confidence interval in brackets.
[2] No mean values provided for test.

Any standardized toxicity test protocol should specify the desired pH range and how to correct pH, if necessary.

4.11 COMPARISON OF TOXICITY TEST RESULTS

The ranges of results for each toxicity test are presented in Tables 4.23 and 4.24. The centrifugate results in Table 4.23 present the trend where Sample A from the DEA plant is most toxic and Sample B from the sulfinol plant is least toxic, with some overlap between Samples B and C. (Sample C being the MEA plant sludge). Examination of Table 4.23 indicates that the sulfinol plant sample (B) is two to six times less toxic than the MEA sample (C), which is in turn five to twenty-five time less toxic than the DEA plant sample (A). It is important to recognize that these conclusions are being reached by different laboratories with different organisms and each type of organism is quite likely responding to different toxicants. Therefore, it is reasonable to say that the DEA plant sample is likely more hazardous to a wider variety of organisms than either the sulfinol or MEA sample and greater caution should be taken in the treatment and disposal of the liquid phases of the DEA sample.

As seen in Table 4.24, the trend in sample toxicity is not as clear for the terrestrial toxicity tests. Sample A is still the most toxic. However, there is some overlapping of toxicity ranges for the seed germination results for Samples B and C while the earthworm results indicate that Sample C is clearly less toxic than Sample B.

The aquatic and terrestrial toxicity tests all generally present acceptable levels of reproducibility, as indicated by coefficients of variation (CV) of less than approximately 25 percent. Where CV values in excess of 25 percent have been seen, review of the data has shown that the discrepancy could have been related to non-homogenous samples, lack of adherence to test procedure and/or variations in test result interpretations.

As the toxicity test results are related to the types of toxic contaminants in each sample, a review of the Phase IIA project inorganic contaminant results would need to be conducted before further discussion of the most probable toxic contaminants could be prepared.

Table 4.23. Range of Centrifugate (Aquatic) toxicity test mean results.[1]

Toxicity Test[2]	DEA Plant (Sample A)	Sulfinol Plant (Sample B)	MEA Plant (Sample C)
Daphnia (n = 3)	LT[3]0.1-2.3	17.7-36.1	0.2-13.5
Algae (n = 2)	0.2-0.8	25.2-29.5	12.3-22.4
Microtox 15min (n = 3)	0.2-0.9	20.7-42.4	4.5-24.5

[1] Greatest sample toxicity values are underlined. Excludes SOS and Toxi-Chromotest results.

[2] EC_{50} or LC_{50} values: n = number of laboratories.

[3] LT = Less than.

Table 4.24 Range of Sludge in Soil (Terrestrial) toxicity test mean results[1]

Toxicity Test[2]	DEA Plant (Sample A)	Sulfinol Plant (Sample B)	MEA Plant (Sample C)
Cucumber germination (n = 3)	2.1-8.5	18.3-36.7	7.8-37.4
Barley germination (n = 3)	1.0-4.6	3.5-28.6	19.5-36.0
Earthworm (n = 2)	LT 0.6	3.0-3.2	10.0-25.7

[1] Greatest sample toxicity values are underlined.

[2] EC_{50} or LC_{50} values: n = number of laboratories.

[3] LT = Less than.

4.12 DISCUSSION OF POTENTIAL TOXICANT SOURCE IN SLUDGE SAMPLES

Chemical analyses provide identification of sample components and approximate distributions. This identification, however, can not tell the analyst whether or not the sample is toxic: only a biological measure can tell you this. Toxicity tests measure the toxicity of a sample on an organism, whether the toxicity is due to an individual toxic component or to a synergism between a number of constituents within the sample.

The organic contaminant analytical results for centrifugate and whole sludge are summarized in Table 3.29. The toxicity test results are summarized in Tables 4.23 and 4.24. Contaminants within each of the organic fractions and chemical classes listed in Table 3.29 could be contributing to the acute toxic effects observed with the aquatic and terrestrial toxicity tests.

When the organic chemical and toxicity test results are compared, a relationship can be seen with the most toxic sample (A) and the highest total sample concentrations (A), both for centrifugate and sludge samples. The variation in the sludge sample ranking may be related to the lack of a chloroform fraction for Sample C sludge. This fraction would contain hydrophobic toxicants, such as dibenzothiophene and phthalate observed in Sample A and B sludges (Tables 3.16 and 3.17), and absence of this fraction appears to have decreased this sample's toxicity. Information on the inorganic chemical composition of the samples must also be considered when attempting to relate chemical components to observed toxicity.

More source-specific toxicity information could be obtained with a closely integrated chemical and biological testing in order to determine which constitutents are responsible for a given toxicity test result. Various toxicant identification schemes have been developed (Poulsen et al, 1984; Mount and Anderson-Carnahan, 1988; Fava et al, 1989) where systematic chemical fractionation/toxicity test detection techniques are used to assist in identifying the predominant toxic contaminants of complex environmental samples. The toxicity identification evaluations usually start with simple testing on the total sample (e.g. pH adjustment, filtering, chelating agent addition, etc.). If warranted the next level of investigation involves chemically separating the different contaminant fractions into discrete fractions which are assessed for toxicity contribution using toxicity tests. Further fractionation and toxicity testing continues only with contaminant fractions where acute toxicity has been observed.

4.13 INTERPRETATION OF TOXICITY TEST RESULTS

In most toxicity tests, the toxic effect is quantified by exposing the organisms to dilutions of the sample and estimating the concentration required to produce a response in 50% of the exposed organisms over a specified time period. The quantified test end-point can be either a LC_{50} (concentration lethal to 50% of the organisms) or an EC_{50} (concentration providing a specific effect in 50% of the organisms).

Toxicity tests integrate the effects of exposure of biological organisms to all the constituents of a sample and measure the type and magnitude of the response after a given period of time. Toxicity data can demonstrate a degree of reproducibility comparable to results of chemical analyses. Also, quality assurance/quality control (QA/QC) programs similar to those for chemical laboratories can be put in place for toxicity testing programs.

Different organisms respond to different toxicants at differing concentrations, due to their respective sensitivities. This sensitivity is dictated by such factors as enzyme systems, metabolic rates, surface uptake rates and rates of internal adsorption of chemicals. The differential organism sensitivity can be used to provide useful information for hazard assessment of samples by using a range of representative organisms that would likely be exposed if the material was introduced into the receiving environment. In the Phase IIB project, three aquatic and two terrestrial tests were incorporated into the toxicity testing battery. A multi-species test program, using organisms representative of separate trophic levels in the receiving environment, would provide a broad information base for contaminant characterization and environmental protection requirements.

The intent of aquatic and terrestrial toxicity testing is to provide toxic effect information that can readily be interpreted into practical terms. One method of more clearly visualizing the degree of acute lethal or sublethal effects is to convert the LC_{50} or EC_{50} to Toxic Units (TU). The TU is derived by dividing the LC_{50} or EC_{50} into 100. The higher the TU number, the more toxic the sample being tested. The TU also clearly identifies the degree of toxicity reduction required to make a sample non-lethal. For example, a sample with a LC_{50} of 5% would have a TU of 20 (100 divided by 5). The TU's of the centrifugate samples are presented in Table 4.25 while those of the sludge samples are presented in Table 4.26. Examination of the Toxic Unit values puts the degree of acute toxicity observed into perspective from an order-of-magnitude point of view. For example, several orders-of-magnitude difference for the DEA plant (Sample A) centrifugate contaminants (as compared to Samples B and C's Toxic Units) clearly indicate that a substantial degree of toxicity reduction would be required before this liquid could be released to the environment.

Although demonstration of techniques for the identification of the toxic chemical components was not within the scope of this current study, the authors would like to make the statement that more causative toxicant information could be obtained with more closely integrated chemical and biological testing schemes, such as those suggested by Mount and Anderson-Carnahan (1988), Hill (1986) and Poulson et al. (1984).

In order to evaluate sludge disposal options, the data required would be comparable to that generated by this study. Decisions should be made on a number of evaluations and not a single data set. It may even be critical to conduct sublethal tests to estimate longer term effects to representative organisms in the

Table 4.25. Comparative toxicity of centrifugate samples.

Organism	Laboratory	Toxic Units Sample Site* DEA Sample A	 Sulfinol Sample B	 MEA Sample C
Daphnia magna (LC_{50})	BQ	40000.0	5.6	200.0
	EPA	43.5	2.8	500.0
	EC - (Edmonton)	142.9	2.9	7.4
Average		13395.4	3.8	235.8
Algae (EC_{50})	EPA	476.2	4.0	8.1
	EC - (Edmonton)	125.0	3.4	4.4
Average		300.6	3.7	6.3
Microtox (5 Min EC_{50})	BETA	111.1	4.0	4.1
	EC	500.0	4.8	5.3
	ERCB	142.9	2.4	6.3
	ESSO	125.0	N/A	22.2
Average		256.0	3.6	11.3
Microtox (15 Min EC_{20})	BETA	500.0	23.3	20.4
	ERCB	333.3	14.7	50.0
	ESSO	333.3	5.3	100.0
Average		388.9	14.4	56.8

* Toxic Units = $100/LC_{50}$ (% v/v) or $100/EC_{50}$ (% v/v).

Table 4.26. Comparative toxicity of sludge samples.

Organism	Laboratory	Toxic Units Sample Site*		
		DEA Sample A	Sulfinol Sample B	MEA Sample C
Cucumber (EC_{50})	AEC	40.0	2.7	4.8
	BETA	47.6	3.3	2.7
	EPA	14.7	5.5	12.8
Average		34.1	3.8	6.8
Barley (EC_{50})	AEC	21.7	3.5	5.1
	BETA		28.6	2.8
	EPA	26.3	5.3	4.5
Average		24.0	12.4	4.1
Earthworm (IC_{50})	BIOQUEST	-	33.3	-
	EPA	-	31.3	3.9
Average		-	32.3	3.9

* Toxic Units = $100/EC_{50}$ (% v/v) or $100/LC_{50}$ (% v/v).

receiving environment. The following example evaluation therefore represents an approach that could be taken to estimating environmental effects.

In order to protect biota against possible lethality from the DEA sludge, application of more than a 100-fold reduction of the liquid phase toxicity would be required, while a 24- to 35-fold toxicity reduction of the solid phase would be required to protect terrestrial life.

The sulfinol centrifugate would require considerably less toxicity reduction (about four to fourteen fold) but the solid phase terrestrial toxicity testing shows somewhat greater toxicity to earthworms than plants. When establishing sludge application rates for land treatment disposal, the different sensitivities of invertebrates (e.g. earthworms) and plants (e.g. forage cover or crops) must be considered. The MEA plant sludge tested in this project would require toxicity reduction equivalent to sulfinol for disposal on land to protect terrestrial invertebrates and crops but leaching of the liquid phase would require more than a ten-fold toxicity reduction to be non-lethal in water. The three samples have clearly distinct and different toxicological characteristics and consequently require different treatment and disposal considerations.

It is important to realize that the conclusions derived regarding relative toxicity and the degree of toxicity reduction required were developed without considering the chemical composition of the samples. The number of chemicals present, their respective concentrations, solubility, sorption coefficients to the sludge matrix, or their chemical/physical/toxicological interactions were not included in the evaluation of toxicity reduction. Decommissioning and clean-up of sites often dicates that specific chemical concentrations be achieved. Any toxicity-based program would require sufficient testing to be consistent with regulatory criteria.

4.14 QUALITY ASSURANCE IN TOXICITY TESTING

In any toxicity assessment, it is critical to have a well-established quality assurance program.

Standardized quality assurance (QA) requirements have not yet been established for toxicity laboratories, although various components are gradually being implemented in laboratories throughout North America and Europe. A number of quality assurance elements were incorporated in this study and were particularly valuable in identifying different practices among labs and establishing a level of conformity. Many of the methods established in chemical laboratories are transferable to toxicity laboratories.

The following overview of toxicity laboratory QA has been summarized from a recent report by Hart (1989). This document has been prepared in recognition of the need for greater QA in toxicity testing, particularly in the framework of regulatory controls.

The elements of a good QA program can be roughly categorized as follows:

- responsibilities and authorities,
- sampling procedures,
- toxicity testing procedures,
- QA/QC procedures, and
- documentation procedures.

Responsibilities and Authorities

Responsibilities and authorities are essential in a QA program for toxicity laboratories. These should be documented in a Quality Management Plan (QMP) or as part of the procedures in a Standard Operating Procedures (SOP) document, and should be clearly assigned and understood by all staff. If QA functions are not clearly assigned, they can be assumed to be the responsibility of someone else and may be sacrificed to budget or schedule constraints.

Sampling Procedures

Proper sampling procedures are critical in the toxicological evaluation of environmental samples. For example, continuous, flow-proportional sampling is necessary to obtain "representative" samples of a discharge with fluctuating or cyclical output. As with all aspects of laboratory QA, good housekeeping and recordkeeping are essential. Sampling equipment should be maintained and calibrated, as required, and efforts taken to prevent cross-contamination of samples. Holding time of samples prior to testing should be minimized.

Toxicity Testing Procedures

Separate areas of the laboratory should be dedicated to sample receipt, reagent storage, sample storage, sample preparation and sample testing. Samples should be logged into a data storage system on arrival with client name, sample name, sample date and sample number. New reagents and instruments should also be logged and labelled, with expiry or maintenance dates recorded. Copies of test methods should be available near the testing area. Bench sheets should be kept in a convenient, organized location during testing, and should be bound for subsequent storage.

QA Procedures

QA procedures should involve a range of activities such as QC testing, knowledge testing and training sessions, review of data prior to release, review of other laboratory records (e.g., log books, reagent labels, etc.), and participation in interlaboratory comparison studies. Records should be kept on the source and date of receipt of test organisms, organism health, acclimation periods, reproductive performance and dilution water characteristics. Test methods should be well-documented in SOPs and preferably follow a recognized standard protocol (e.g., Environment Canada, U.S. EPA, ASTM, OECD Protocols).

Quality Control

All tests should be run with appropriate control solutions (e.g., blanks) in which test organisms should demonstrate good survival. Acceptable mortality is typically less than 10%, although some test procedures allow up to 20%.

Reference toxicant tests should be performed for each test type and test organism on a regular basis as a general measure of test reproducibility. Control charts should be established to track reference toxicant results over time with respect to established control limits to allow immediate identification of out-of-control conditions. Data which are out of control should immediately be reviewed to identify the source (if possible) of variability (Environment Canada, 1990b).

Replicate tests should be performed routinely, in order to quantify the within-lab precision or reproducibility associated with individual samples. Range-type control charts similar to those used by chemical laboratories can be established to track duplicate test performance using effluent samples or reference toxicant solutions.

Interlaboratory testing is important to identify comparability of results between laboratories. Either reference toxicant solutions or toxic effluent samples can be split among laboratories and the results can be used to estimate interlaboratory test variation. With a sufficient number of participating laboratories, tests can also be performed to identify laboratories which consistently report outside the normal range of interlaboratory variation.

As discussed in another section of this report, reference toxicants were valuable in identifying procedural differences among laboratories and conformity among labs was improved. Whenever significant differences in test results are identifed among laboratories, there is a need to investigate and attempt to identify the cause of the differences. The final analysis may result in evidence to justify laboratory differences (e.g., species stock sensitivity) as opposed to discrediting or rejecting data.

4.15 COST-EFFECTIVENESS OF VARIOUS TOXICITY TESTS

The project costs per test and the number of required tests per sample are presented in Table 4.27. Daphnia and the algal toxicity test both appear to be the least expensive tests for a sample analysis. The algal bioassay requires the additional expense of a cell counter but this results in a significant increase in sample testing capability plus increased accuracy, especially at low cell counts. The Microtox bioassay is somewhat more expensive and has the additional expense of requiring very specific equipment.

Of the terrestrial tests, the earthworm is less expensive, even when considering the additional cost of soil preparation. The seed germination test would be comparable if only one seed species was analyzed.

Aquatic toxicity test costs derived for another study (G. Sergy, 1987) are presented in Table 4.28.

Although the costs presented in Table 4.28 are based on different assumptions than those in Table 4.27, it is evident that the Daphnia and the Selenastrum (microplate) aquatic toxicity test are cost-efficient.

In general, the aquatic toxicity tests are more economical than terrestrial tests and, combined with the observed test results in previous sections, also appear quite reproducible among laboratories if proper protocol standardization is performed.

Table 4.27. Individual toxicity testing costs for Phase IIB.

Toxicity Test	Cost per Test	Number of Tests per Sample	Sample Analysis Cost	Additional Costs/ Special Requirements Not Included in Test Cost
Daphnia	\$ 75	5[1]	\$ 375	Controlled environment chamber
Selenastrum	\$ 75	5[1]	\$ 375	Controlled environment chamber: cell counter
Microtox	\$ 92	5[1]	\$ 460	Specific Microtox equipment and solutions
Eisenia	\$300	5[1]	\$1500	Preparation of the soil/sample dilutions (\$750)
Seed Germination	\$ 50	62[2]	\$3100	Controlled growth chamber

[1] Includes three replicates and two reference compounds.
[2] Includes six concentrations and one blank in three replicates, for two seed types; plus range finding for 6 concentrations and one blank for two seed types; plus triplicate analysis of reference compounds for two seed types.

Table 4.28. Canadian private sector toxicity testing costs (1987\$).

Toxicity Test	Cost per Test Based on 10 Test Batch Average
Daphnia,	
48 hour static	\$200
Selenastrum,	
4 day microplate	\$125
8 day conventional	\$600
Microtox	\$100

From G. Sergy (1987).

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

- The pond sampling procedure resulted in representative sludge and centrifugate composite sample collection. Pre-sampling site reconnaissance trips and testing by technical personnel familiar with the method are essential.

- Sample homogeneity appeared to be a problem for test reproducibility when samples containing more than one liquid phase (one being an oil phase) were tested.

- Unacceptable toxicant modification or degradation can also be experienced when samples are being transported to the testing laboratory. It is critical that testing be performed on fresh samples and that transportation delays be documented. As sample degradation due to shipment problems is a potential major source of analytical variability, sample transport and handling procedures must be rigidly controlled.

- The organic chemical fractionation method utilized by ENVIRO-TEST effectively divided the samples into chemical subgroups.

- The use of calibration compounds to standardize the alumina column is essential for inter-laboratory performance comparison. Adequate quality control measures are also necessary.

- The lack of agreement in the results between ENVIRO-TEST and KCER suggests that the fractionation and identification protocol requires further standardization to ensure reproducibility, as well as establishment of a definitive method for analysis of multiphase samples.

- The identification of fraction chemical components of sludge or centrifugate fractions alone does not result in an explanation of the source of sample toxicity.

- The use of reference toxicants proved effective in procedure standardization among participating laboratories.

- The three aquatic toxicity tests (algae, Daphnia and Microtox) performed comparably well. Both the algae and Daphnia assays generally had within-sample coefficients of variation under 20%. The algae assay was the most consistent with respect to within- and among-laboratory variation, even though two variations of the method were used by the two laboratories. Although within-laboratory sample variation was fairly uniform for the Microtox test, most laboratories showed distinct differences in the recorded average levels of toxicity, i.e. accuracy. With the exception of ESSO, the within-laboratory sample variation for Microtox assays were under 40% for both EC_{20} and EC_{50} tests. Statistical among-laboratory differences in the Microtox assay results were least for the EC_{20} tests, probably as a result of the higher sample variability (standard deviation) observed in comparison to the magnitude of the mean.

- Of the two terrestrial toxicity tests (seed germination and earthworm), the seed germination test appeared the most variable in terms of within- and among-laboratory variation, although minimal information was available to fully assess the reproducibility of the earthworm test. Two seed types were used in the germination assay, cucumber and barley. Barley exhibited less within-laboratory variability, and a somewhat higher sensitivity.

- The aquatic toxicity tests were generally more sensitive to the gas plant centrifugate samples than the terrestrial tests of the corresponding sludges, as indicated by lower values for EC_{50} or LC_{50}'s for corresponding samples. The earthworm test appeared quite sensitive but insufficient results were obtained to make a definitive conclusion.

- For both the terrestrial and aquatic toxicity tests, within-sample variation tended to respond inversely to increasing potential toxicity, e.g., Sample A, the most highly toxic, tended to have the lowest associated coefficients of variation as compared to Samples B and C. This observation could be partly due to the test population genetic variability when responding to stress conditions, i.e. when the toxicant is not so toxic to kill virtually all of the population quickly (low EC_{50}'s values), then some members of the population may be flexible enough to adjust to lesser levels of toxicity, generating more variablity. An exception to this observation is evident with the Microtox results (ESSO's excluded), where the most toxic sample (A) now shows the highest levels of variation (highest CV values) and the least toxic sample (B) shows the lowest variation.

- Based on the small number (3) of replicates per sample, it is difficult to determine whether the observed within- and among-laboratory variation is a consequence of non-standardized protocols, or is simply reflective of the normal test organism and procedure variation for a particular biological test. One possible way to overcome this problem is to establish an impartial laboratory where all tests are replicated sufficiently, using selected reference toxicants, to determine the expected and acceptable variations in response. The reference toxicant toxicity values obtained from other laboratories could then be compared to the standards set by the control laboratory, and re-evaluation required if the values lie beyond acceptable levels. This would provide quality control over both the test organisms used and the procedures employed.

- The Microtox test, even though a very standardized protocol, presented variations possibly the result of some laboratories extrapolating EC_{20} and EC_{50} values from the data. This practice provides unreliable results which, as shown, decrease the reproducibility of toxicity result among companies.

- The most variable results were observed with the seed germination test. Barley exhibited less variability and greater sensitivity than cucumber, but inter-laboratory reproducibility was not as good as with most of the other toxicity tests.

- Due to the procedural problems encountered in the earthworm toxicity test, limited information was obtained from the two participating laboratories. The test procedure was the most time-consuming of all the toxicity tests undertaken, from a sample preparation standpoint. This could lead to reproducibility problems. Another major problem observed with this test was the wetness of the sludge samples. The defined water-holding limit for the test could not be met at certain sample concentrations. At lower sample concentrations, there was concern over homogeneity of mixing. These points must be considered to improve the reproducibility of test results.

- Statistically similar inter-laboratory results generally agree with overlaps of the 95 percent confidence intervals, from a coarse point of view. In some cases, though, the statistical evaluation can identify equities or inequities of results that otherwise are not identifiable by confidence interval comparison.

- Statistically-derived within-sample significant differences that indicate potential problems with data reproducibility do not, however, interpret directly into biological significance. Laboratories may report statistically different toxicities for a sample but biologically the toxicity may be the same. Conversely, some results may be statistically similar to other results based on their large variability, but comparison of the means and confidence intervals indicates there may be different biological impacts. The interpretation of the results should be dictated by the specific concern applicable to any given sample analysis, i.e. reproducibility of results by laboratories or sample toxicity to a biological system.

5.2 RECOMMENDATIONS

5.2.1 Sampling and Distribution Recommendations

The following sections outline specific recommendations arising from the sampling and sample preparation components of this study.

Sampling

- It is recommended that all sludge containment sites be visited prior to sampling to develop appropriate sampling methodology and to obtain a preliminary sample for initial characterization.

- The use of a crane outfitted with a man-basket proved very efficient for the collection of sludge samples from small ponds and should be considered in the planning of sludge collection protocols. Utilizing a two-man sampling crew, a large representative sample (120 L) could be obtained, under good weather conditions, in a single working day.

- A light core sampler, consisting of aluminum or polyethylene tubing and capped with an air tight seal, proved effective for the collection of most sludges and could be modified with a cork capping for less consolidated sludges. However, water depths exceed one to two metres and sludge depths are shallow (less than 0.5 m), a dredge may prove more effective for sample collection than a core sampler.

- The CPA sludge sampler would not retain the sludge sample without the use of a core catcher and is very heavy and cumbersome to use. It did not prove effective for the less consolidated sludges sampled and is not recommended for use unless significantly modified.

Personal Protection

- Personal protection equipment worn during this study is considered adequate for the protection of sampling personnel. However, care should be taken to tape all seams, and open areas (i.e., around boots, gloves and hoods) to minimize the potential for direct skin contact with sludge material.

- The quantity of sample required and the depth of the ponds in this study increased potential exposure of sampling personnel through splashing of sludge as it was removed from the core sampler. The use of full-face respirators and Saranex-coated Tyvek suits equipped with hoods is recommended. Care should be taken to tape all seams (around face masks, boots, gloves and hoods) to minimize the potential for direct skin contact with sludge material.

Sample Preparation and Distribution

- The volume of sample required for this study created problems for sample homogenization. Attempts should be made to reduce the sample quantity as much as possible.

- Protective equipment should be worn during the sample homogenization process.

- Preparation of centrifugate using laboratory-sized centrifuges is not recommended for large volume samples. The process is time-consuming, tedious and exposes laboratory personnel to sludge vapours and liquids over relatively long periods of time. Tests should be undertaken to compare the chemical composition of the aqueous phase of the decanted liquids to that of the centrifugate to determine if this liquid would provide the same information, thereby eliminating the need to centrifuge. However, preparation of smaller volumes of centrifugate (1 litre) with laboratory-sized centrifuges, for chemical and toxicity test screening, should still be cost-effective.

- Testing should be undertaken to determine if, and to what extent, the homogenization and subsampling procedures result in significant losses of volatile organic components.

- All samples must be packaged and shipped to laboratories following TDG Regulations. If samples must be shipped to the U.S. for analysis, as in this case, all reasonable efforts must be expended by shipper and receiver to expedite transport of samples.

- Liquid samples should be assessed to determine whether they are multiphasic. If multiphasic, the central laboratory should consider separating the different phases prior to distribution to the external laboratories

- After preparation and prior to distribution, the accuracy of reference toxicant test stock solution concentrations should be assessed by chemical analysis and be labelled accordingly. Dilutions of stock solutions at participating laboratories should also be analyzed.

5.2.2 Organic Analyses Recommendations

- Both the sample fractionating column and the screening analysis must be validated through the use of calibration compounds, as well as surrogates. The results of this phase of the study show, from the surrogate recovery information, some problems with reproducibility of results which can be traced to the quality of the fractionation column. The use of calibration compounds would provide a test of the fractionating column's capability of adequate chemical class separation.

- The specifications of inter-laboratory recoveries for deuterated surrogates of greater than 50% are generally achievable. Only ENVIRO-TEST presented relative standard deviations and these (see Table 3.4 and Table 3.6) generally meet the reproducibility criterion of better than 20%. It is recommended that laboratories provide relative standard deviations for deuterated surrogate results, or some other index of variability, as an indicator of the reproducibility of the procedure.

- To further reinforce the reproducibility of a procedure, it is recommended that three to five laboratories develop a range of acceptable data with respect to surrogate recoveries and the range of specific components to be identified. This may require a further standardization of the procedure, centralization of an adsorbent source to minimize column variation and specification of the method to be used for compound identification, eg., specify library or method of manual interpretation for non-target compounds.

- Another aspect of the reproducibility question is the need for a quality control protocol. This would be a standardized and regimented set of procedures that could generate results that provide a secure basis of comparison with other laboratories.

- To provide sufficient data to enable assessment of risk with respect to classification and handling of waste, a balance must be achieved between the needs for chemical information and environmental significance. The specific compound GC/MS evaluation provided a chemical profile of the sample but lacked considerably when it came to interlaboratory reproducibility and economic frugality. Similarly, initial screening of the fractions only permitted a rough analytical segregation of general groups of compounds without precise evaluation, but with the possibility of better reproducibility. The decision must be made as to what type of balance is necessary between specific chemical composition and biological toxicity that will provide adequate information to safely deal with classification and/or treatment assessments of complex waste materials. -

- The organic analyses should be integrated with the toxicity testing if information relating composition and biological toxicity is to be obtained. Coarse initial tests could be done on the entire sample, both chemical and biological, to help identify the type of specific testing that would be most appropriate. This would be followed by sample fractionation and further toxicity tests. A tiered and integrated testing procedure such as this would minimize costs, since only toxic fractions would be further analyzed. This method would permit identification of the toxicant(s) more clearly and support remediation decisions more effectively. The choice of the toxicity tests to be used would be determined by considering the possible disposal/remediation alternatives and their most likely routes for toxicant exposure.

5.2.3 Toxicity Testing Recommendations

The toxicity test portion of this study has assisted in further defining critical factors when considering biological testing as a means of characterizing gas plant sludges. This involves parameters dependent on sample characteristics, project management, statistical evaluation, test protocols and quality control. The following sections outline the specific recommendations arising from the Phase IIB study.

Sample Dependent Factors

- While considerable effort was expended in attempting to generate homogeneous sludge samples, the heterogeneity of the gas plant sludges collected presented some problems. Some samples were oily wastes which separated into different phases upon settling. Actions were taken during this study to counter this problem, i.e. decanting of the surface oil phase prior to testing. These measures, however, were not adequate to prevent problems and procedures must be established and incorporated into the testing to accommodate this problem. It is recommended that the primary laboratory conduct any pre-testing steps, such as decanting or centrifuging, prior to distribution to an external laboratory for analysis.

- Delays in transportation and testing result in changes in potential toxicants due to various forms of degradation (biological degradation, photolysis, hydrolysis, etc.), leading to different toxicity determinations among laboratories. Adequate preparation and planning must be undertaken in order to prevent this.

- The issue of pH and sample hardness adjustment should be addressed in the appropriate test methods. In this study it was decided that, if sample water hardness for the *Daphnia* test was below 80 mg/L $CaCO_3$, hardness was adjusted up to 200 mg/L, since the *Daphnia* were acclimated to this level. The effect of pH adjustment was briefly investigated and it was apparent that adjustment of pH can cause a change in the detected toxicity level. The degree of effect due to pH adjustment varied among toxicity tests. These preliminary test results indicated that, for a pH change from 8.24 down to 7.04, the EC_{50} value increased approximately 50 percent for Microtox (15 minute). The effect on the algae tested is inconclusive but appears to be reverse to that observed with Microtox.

- When the seed germination test is being considered for evaluation of sludge toxicity and potential disposal alternatives, it is suggested that one of the species to be tested be chosen from representative species in the area of potential contamination. These tests could also report root elongation to monitor developmental toxic plant effects.

Project Management

- The results of this study reinforce the need in future methods validation efforts to place particular attention on test coordination. This would include communication of concise test procedures, compilation of complete test results, standardization of the appropriate method of analysis and evaluation of the results. If the results are unsatisfactory, re-testing requirements would be specified. Communication, as well as the ability to obtain the modifications requested, is of utmost importance.

Procedure Standardization

- Increased standardization of toxicity test procedures could be accomplished by further definition of test method variables and determination of the test limits, such as reference toxicant acceptable ranges, which could then be used to assess laboratories' performance with respect to reproducibility and acceptability. The use of five replicates, rather than three, is also recommended on the basis of statistical considerations. The standardized protocol should also define a source of test organism stock, acclimation period, reference toxicant to be used for test performance appraisal, and information sources when clarification is needed. These are all components of a laboratory's Standard Operating Procedure (SOP). This type of protocol-related information is similar to that being prepared by ENVIRONMENT CANADA (Environment Canada, Aquatic Biological Test Method, May 1989).

Future Considerations

- An integrated, tiered protocol for combined organic, inorganic and toxicity test analyses similar to those discussed by Hill (1986) and Fava et al. (1989) should be attempted to help identify sources of toxicity in a cost-effective manner and to more efficiently connect these two complimentary types of analyses together. Use of such a protocol would consist of coarse initial chemical and toxicity testing to provide information on whether the sample is toxic. Further analysis would occur on fractions generated with the intent to isolate environmentally important chemical classes. This analysis would be of an intermediate level with respect to cost and specific information provided. Toxicity testing on fractions would assist in specifying the chemical class primarily responsible for an observed toxic response. Detailed chemical analysis, even though more costly, would be performed only if inadequate information existed to that point. This integrated method would permit cost-effective identification of the type of biological toxicity and the chemicals responsible for that response. This is valuable and essential information when evaluating the environmental impact of a complex waste and when considering methods of remediation and environment recovery.

- Reference toxicant data should be statistically evaluated to establish the effect of procedural and analytical variations.

- In the future, a battery of aquatic and terrestrial toxicity tests could provide valuable information on the feasibility of certain types of disposal or remediation methods. For example, an area considered for landfill having a relatively high water table or close to a potable water source would not likely be the best disposal option for a sludge exhibiting high toxicity levels during aquatic toxicity testing of the generated centrifugate. Also, the seed germination test can select native varieties from a proposed disposal option area to quickly determine their specific sensitivities to potential contaminants in a given sludge.

- This study concentrated on acute lethal and sublethal responses since the tests are quick to perform, with a well defined end-point. These tests provide important information about waste characterization. However, it is recommended that the final battery of tests include at least one genotoxicity test, such as the Ames test or S.O.S. Chromotest, which is capable of identifying potential mutagenic or carcinogenic effects of a waste. If this is pursued, comparison testing between the Ames test, S.O.S. Chromotest and the new Microbics genotoxicity screening procedure on a variety of wastes could assist in choosing the most reproducible, informative, and cost-effective test.

6. ACRONYMS

ANOVA	analysis of variance
B/N	base/neutral fraction
BHEED	N, N' - bis (2-hydroxyethyl) ethylene diamine
BHEP	N-N' - bis - (hydroxyethyl) piperazine
BTEX	benzene/toluene/ethylbenzene/xylene
$CaCO_3$	calcium carbonate
C:N	carbon to nitrogen ratio
CPA	Canadian Petroleum Association
CV	coefficient of variation
DCM	dichloromethane
DEA	diethanolamine
DEG	diethylene glycol
DIPA	diisopropanolamine
EC	Environment Canada - Environmental Protection
EC_{20}	concentration of test material causing inhibition of growth to 20 percent of the population
EC_{50}	concentration of test material causing inhibition of growth to 50 percent of the population
EPA	Environmental Protection Agency (U.S.)
FMOC	9-fluorenylmethyl chloroformate
GC/FID	gas chromotography/flame ionization detection
GC/FPD	gas chromatography/flame photometric detection
GC/MS	gas chromatography/mass spectrometry detection
GC/NPD	gas chromatography/nitrogen phosphorus detection
HEED	N - (hydroxyethyl) ethylenediamine
HEM	N - (hydroxyethyl) ethylenimine
HEP	N - (hydroxyethyl) piperazine
HPLC	high performance liquid chromatography
KCER	Kananaskis Centre for Environmental Research
LC_{50}	concentration of test material lethal to 50 percent of the population

MEA	monoethanolamine
MW	molecular weight
OX	oxazolidone
PAH	polycyclic aromatic hydrocarbons
PANH	polycyclic aromatic nitrogen heterocyclics
PASH	polycyclic aromatic sulphur-containing heterocyclics
QA/QC	quality assurance/quality control
SOP	Standard Operating Procedure
TDGR	Transportation of Dangerous Goods Regulations
TEA	triethanolamine
THEED	N, N, N' - tris - (hydroxyethyl) ethylenediamine
TKN	total Kjeldahl nitrogen
TOC	total organic carbon
UV	ultra-violet

7. REFERENCES

Blaise, C. 1984. Developpment de bioessais subletaux pour les evaluations ecotoxicologiques des effluents. Theses de Doctorat en toxicologie de 1,Universite de Metz (France), No. 4, 172 pages.

Blaise, C., R. Legault, N. Bermingham, R. Van Coillie, and P. Vasseur. 1986. A Simple Microplate Algal Assay Technique for Aquatic Toxicity Assessment. Toxicity Assessment : An International Quarterly Vol. 1, 261-281.

Brusick, D.J. and R. R. Young. 1982. IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. U.S. EPA Report. NTIS PB 82 228/966. 5285 Port Royal Road Springfield, VA.

Clonetics, 1986. The Blue S.O.S. Chromotest Kit. Two Step Version 2 (United States). Instructions for Use.

Einarsson, S., S. Folestad, B. Josefsson and S. Logerkuist. 1986. High-resolution reversed-phase liquid chromatography system for the analysis of complex solutions of primary and secondary amino acids. Anal. Chem. 58 : 1638-1643.

Environment Canada. May 1990a. Acute Lethality Test Using Daphnia sp. Biological Test Method Series. ESP/5/90/101.

Environment Canada. 1990b. Guidance Document on Control of Aquatic Toxicity Test Precision using Reference Toxicants. EPS/5/90/103.

Fava, J.A., D. Lindsay, W.H. Clement, R. Clark, G.M. DeGraeve, J.D. Cooney, S. Hansen, W. Rue, S. Moore, and P. Lankford. 1989. Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations (TRES). U.S. EPA Report EPA-600/2-88/070.

Federal Register. 1984. Methods 1625 and 625. Volume 49:184-189 and 153-174.

Fender, W.M.. 1985. Earthworms of the Western United States. Part I. Lumbricidae. Megadrilogica 4(5): 93-129.

Greene, J.C., S.A Peterson, C.L. Bartels and W.E. Miller. 1988. Bioassay Protocols for Assessing Acute and Chronic Toxicity at Hazardous Waste Sites (Draft Protocol). U.S. Environment Protection Agency.

Hamilton, M.A., R.C. Russo, and R.V. Thurston. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11(7): 714-719.

Hart, D.R. 1989. General QA/QC Guidelines for Bio-environmental Studies. Beak Consultants Limited. Prepared for Environment Canada, Inland Waters Directorate, Burlington, Ontario.

Hill, S.L. 1986. Toxicological Characterization of Water Produced During Simulated In-Situ Processing of Green River Formation Oil Shale. Report for U.S. Department of Energy, Laramie, Wyoming.

Miller, W.E., J.C. Greene, and T. Shiroyama. 1978. The Selenastrum capricornutum Printz Algal Assay Bottle Test: Experimental Design, Application, and Data Interpretation Protocol. EPA-600/9-78-018, U.S. Environmental Protection Agency, Corvallis, OR.

Monenco Consultants Limited. 1985. Characterization of Sludges Associated with Natural Gas Processing. Phase I. Prepared for the Environmental Research Advisory Council of the Canadian Petroleum Association and the Environmental Protection Service of Environment Canada.

Monenco Consultants Limited. 1987. Preliminary Field Validation of Methods for Determining the Characteristics and Environmental Significance of Natural Gas Processing Plant Waste Sludges. Phase II. Prepared for the Environmental Research Advisory Council of the Canadian Petroleum Association and the Environmental Protection Service of Environment Canada.

Mount, D.I., and L. Anderson-Carnahan. 1988. Methods for Aquatic Toxicity Characterization procedures. U.S. EPA Report EPA-600/3-88/034.

Nasholm, T. , G. Sandberg and A. Ericsson. 1987. J. Chromatography 396 : 225-236.

Orgenics Ltd. 1986. The Toxi-Chromotest: Instructions for Use.

Porcella, D.B. 1983. Protocol for Bioassessment of Hazardous Waste Sites. U.S. Environmental Protection Agency Report EPA-600/3-83-054.

Poulsen, R.E., Hill S.L., Mason, J.M. 1984 Chromatographic Profiles of Oil Shale Processing By-Product Waters and Selected Fractions. Prepared for the U.S. Department of Energy

Sergy, G.A. 1987. Recommendations on Aquatic Biological Tests and Procedures for Environmental Protection, C&P, DOE. Prepared for the Department of the Environment.

Sokal, R. and F. Rohlf. 1981. Biometry. 2nd Edition. W.H. Freeman and Company, New York.

Thomas, J.M. and J.E. Cline. 1985. Modification of the Neubauer Technique to Assess Toxicity of Hazardous Chemicals in Soils. Environ. Toxicol. Chem. 4:201-207.

Valdez, D. 1985. Silyation of Dilute Hydroxy Compounds in Aqueous Solutions. J. Chromatographic Sciences 23:128-131

Valdez, D. and J.C. Reier. 1986. A Simplified Procedure for the Derivatization of Alcohols at Dilute Levels in Aqueous Solutions with 3,5-Dinitrobenzoyl Chloride. J. Chromatographic Sciences 24:356-360

APPENDIX A

SLUDGE SAMPLING PLAN

SAMPLING PLAN

A1.0 Sampling Strategy

Each pond will be sampled on a grid basis to recover a composite sample which is considered representative of the total sludge content. The number of individual grid or sample points is determined based on the estimated sludge depth, size of the sampler and overall sample recovery.

Samples will be obtained using the CPA core sampler. This unit consists of a 1.0 m long by 53 mm diameter steel core barrel. The unit is fitted with a removable aluminum sleeve with an internal diameter of 47 mm. The sampler will be extended to 2 m in length and outfitted with a "T" handle of variable length, to permit operation at varying pond depths.

The depth of sludge will vary from pond to pond and by location within a single pond. However, for purpose of estimation, it was assumed that the average sludge depth is 1.0 m. Using this sampler it is estimated that approximately 1.5 L of sludge will be recovered from each sample location. Therefore, a minimum of 60 core samples will be required to generate a total composite sludge volume of 90 L.

Each pond will be measured and subdivided using an evenly spaced grid. The grid dimensions will vary depending on actual pond measurements. Perimeter grid location will be marked with a metal pin and flagging tape.

A1.1 Sampling

Samples will be obtained using a mobile crane outfitted with a personnel basket. One technician will operate the sampler from the personnel basket; the other will recover the samples at a convenient location adjacent to the pond.

The crane will hoist the technician to the first sample location. The core sampler will be inserted and rotated into the sludge as deeply as possible by hand. The "T" handle of the sampler will be attached to the personnel basket, or an overhead crane line, for retrieval. The sampler will be hoisted to the sample recovery area where the aluminum sleeve will be retrieved over an open 20 L sample container. A new sleeve will be inserted in the sampler and the process repeated.

Samples will be obtained in a similar fashion using a 50 mm diameter polyethylene core sampler for the Phase IIA inorganic component of the project. The minimum sample requirement for this component is approximately 10 L. Therefore a minimum of 10 core samples will be obtained; these will be evenly distributed on the existing grid system to yield a representative sample.

A1.2 Sample Handling

All core samples for the Phase IIB project will be composited in a series of five 20 L alloy steel containers. Excess free water will be decanted at the site. Samples for the Phase IIA component will be composited in a single 20 L polyethylene container. All samples will be kept cool and transported to the receiving laboratory as soon as practical. Because of timing, no supplementary refrigeration of field samples will be necessary.

Sample containers will be placarded as required under the Transportation of Dangerous Goods Regulations. Information on sample classification will be verified with the Compliance Information Centre.

A1.3 Sample Preparation

Free water will be decanted from each sludge container and will be stored for potential future use. Sludge within each sample container will be homogenized using an electrically powered mixer. The Phase IIB sludge samples will be sub-sampled at this time using a small diameter (20 mm) glass tube. Approximately 40 sub-samples from each of the five

20 L containers will be taken and composited to form the raw sludge sample which will be used for the organics analysis, seed germination and earthworm toxicity tests. This composite will be homogenized and sub-sampled again, as described, to provide each laboratory with its required sample.

The remainder of the sample will be centrifuged to remove interstitial water. This "centrifugate" will be used for all aquatic toxicity tests. Three seperate centrifuges will be used, each with a capacity of 1.5 to 3 L.

Each centrifuge will be dedicated to one of the three sludges. The sludge will be centrifuged in aliquots and all centrifugate will be combined in a single container. The organic phase (if present) will be removed and stored for potential future evaluation. The aqueous phase will be sub-sampled to provide individual laboratory requirements for the aquatic toxicity tests.

If insufficient centrifugate is generated, the decanted free water will be combined with the sample and the total sample will be filtered prior to sub-sampling.

All samples will be packaged in glass containers and couriered to laboratories. All samples will be labelled with a unique laboratory number; laboratories will not be advised of the sample origin.

A2.0 Personnel Protection

A2.1 Safety

The proposed work plan will be reviewed with individual plant operators/safety personnel to ensure all safety requirements are met. The plant will be advised of the sampling schedule at least one week in advance. All Western Research employees will be required to complete the safety orientation program prior to commencing any work on the plant site. The plant will supply a safety trained observer to maintain surveillance over the sampling program.

A2.2 Personnel Protection Equipment

The following protective equipment will be worn by the sampling crew:

- hard hat
- safety glasses/face shield
- Saranex coated Tyvek coveralls
- steel toed rubber boats
- inner disposable latex gloves
- nitrile gloves
- half face respirator equipped with an acid gas/organic vapour cartridge and particulate filter.

This equipment will provide adequate protection against dermal contact with the sludge material. Respiratory protection is considered adequate for low level organic vapours only.

A2.3 Additional Safety Considerations

The crane and sample recovery area will be established on the upwind side of the pond if possible. If an H_2S risk is expressed by the plant the samplers will don self-contained breathing apparatus (SCBA) and "probe" the pond in several locations to check for a significant evolution of gas. The plant safety co-ordinator or representative will check H_2S levels during this operation. If no significant H_2S levels are observed, sampling will procede using the proposed personnel protection equipment. If significant H_2S is encountered, a supplied breathing air (SBA) system will be employed for the sampling team and crane operator.

Plant procedures for crane operation and personnel baskets will be followed. The technician collecting samples will wear a safety line and may be required to wear a life preserver.

A3.0 SUMMARY

Sampling Procedure

- contact plant and establish schedule
- arrive at plant for safety orientation
- tailgate safety meeting with sampling crew, plant contacts, and crane operator
- prepare work area; establish dirty sample receipt area and lay down 4'x8' sheet of plywood to act as working surface
- mark out grid pattern on pond edge for orientation
- spot crane and rig up personnel basket
- don protective clothing
- one technician to operate sampler from personnel basket, one technician to recover sample in receipt area
- sling basket to first grid point, obtain core sample and sling basket to sample receipt area (steel sampler used for organic samples polyethylene tube used for inorganic samples)
- deposit core in 20 L container (steel container used for organic samples and polyethylene container for inorganic samples)
- repeat procedure for all grid points and/or until required sample volume is obtained
- when sample container is near full, manually mix contents and allow to settle, decant excess free liquid
- seal containers and label with plant identifier, pond type, sampling date and TDGA label
- dispatch crane, clean up samplers and work area
- remove protective gear and dispose of contaminated material according to normal plant practice
- transport containers, maintaining at a cool temperature, to receiving lab

APPENDIX B

TOXICITY TEST PROCEDURES

TOXICITY TEST PROCEDURES

The following toxicity test procedures were presented in the USEPA draft (1988) and were adopted for this study. The Microtox procedure originated from round-robin studies and is presented in this section.

B1 Glossary

This section provides definitions of several technical terms used in the toxicity test procedures.

Acute Toxicity A relatively short-term lethal effect.

Chronic Toxicity Longer-term effects that may be related to growth, metabolism, or reproduction.

Effective Concentration (EC) The concentration of a test sample that produces an effect (not including death) at a specified level, on a specified percentage of the exposed organisms, and within a specified time of exposure.

Elutriate A solution obtained by mixing a solid sample with a specified weight ratio of water prior to separation by centrifugation and filtration.

Eluent Solvent used to extract contaminants from solid samples (ie. solvent used to produce elutriates). Solvent for this protocol is deionized or distilled water.

Elute The process of preparing an elutriate.

Leachate Water that has percolated through a column of soil or waste.

Lethal Concentration (LC) Concentration of a test sample that produces death for a specified percent of the exposed organisms at a specified exposure time.

Sample, Standard A sample of constant or defined composition (eg. synthetic water or artificial soil).

Soil, Artificial (AS) Non-toxic synthetic soil, prepared in the laboratory using a specific formulation, used as a control and diluent medium in the earthworm and seed germination tests.

Soil, Test (TS) A mixture of contaminated site soil and artificial soil used in a toxicity test.

Water Holding Capacity (WHC) The ability of a soil to maintain a water-saturated condition.

B2 General Methods and Sample Preparation (from Greene et al, 1988)

This section presents information on methods and materials that are common to all of the tests and details for the preparation of water, soil and sediment samples.

The procedure recommends the use of only distilled or deionized water for elution of site samples. Mobility of toxic contaminants, whether through the groundwater or off-site from surface run-off, will primarily be caused by rainfall. If solvents are part of the site contaminants they may increase solubility and mobility of certain toxic constituents. However, for the purposes of toxicity tests recommended in this procedure outline, which attempt to define toxicant availability in a more "real" sense, the use of solvents to maximize extraction of the toxic constituents from soils or sediments is unacceptable.

B2.1 Facilities

The facilities used for aquatic toxicity tests should include tanks equipped with temperature control and aeration for holding and acclimating test organisms, a recirculating water bath for the test vessels, or a controlled environment chamber. A controlled environmental chamber is also necessary for conducting the terrestrial tests. If the use of reconstituted dilution water is necessary, there should be a tank for its preparation. Air used for aeration must be free of oil and fumes. The test facility must be well ventilated and free of fumes. Materials that come in contact with site samples, test solutions, or dilution media should not contain substance leach or be dissolved into water. Materials that minimize sorption of sample constituents should be selected. Glass, #316 stainless steel, and perfluorocarbon plastics must be used whenever possible to minimize leaching, dissolution, and sorption. Unplasticized plastics may be used for holding and acclimation tanks and in the water supply system. Rubber, copper, brass, galvanized steel, and lead must be avoided. If stainless steel is used, it must be welded, never soldered. Silicone adhesive used to cement glass containers absorbs some organochlorine and organophosphorus compounds which are difficult to remove. Therefore, as little adhesive as possible should be in contact with the test solutions and extra beads of adhesive should be on the outside, not the inside, of the containers.

B2.2 Cleaning and Preparation of Glassware

Each testing container must be cleaned, by the following method, before use or reuse.

- washed with non-phosphate detergent
- rinsed with 100 percent acetone
- rinsed with 10 percent HCl
- neutralized with saturated sodium carbonate solution
- rinsed thoroughly with tap water or other clean water
- rinsed with distilled or deionized water (3 volumes)
- baked at 300°C for 10 hours to remove residual organic contaminants. Before reuse, each container should be cleaned as above.

B2.3 Test Materials for Water Samples

Test solutions may be surface waters, groundwater, or elutriates prepared from soils or sediments. The following sample volumes are required for the freshwater tests:

Test Species	Sample Volume (L)
Daphnia magna	2
Selenastrum capricornutum	1

Water samples should be filtered (0.45 µm cellulose acetate filters) to remove indigenous organisms and suspended particulates. Filtration must be done before sample storage. Samples must be covered at all times and violent agitation must be avoided. Samples that are to be tested in the laboratory should be placed on ice during transportation. Testing should be performed as soon as possible after receipt of the samples. If testing is not initiated upon receipt of sample, the filtered (0.45 µm) samples should be stored in the dark at 4°C.

The temperature of the sample should be adjusted to that of the test (±2°C) before portions are added to the dilution water. Surface water, groundwater, soil elutriate or sediment elutriate samples are added directly by volume to a dilution media. The dilution medium used depends upon the test that will be performed. Algal assay medium is used to dilute site samples on which algal test are to be performed. Site samples on which Daphnia magna tests will be performed are diluted with de-ionized or distilled water. Aliquots of the site sample are added to the appropriate dilution medium to prepare each replicate test solution. If it is not possible to prepare a homogenous solution of the toxicant, it must be added directly to the dilution medium in each replicate test vessel. Up to 100 percent of the filtered sample can be used for the Daphnia magna and up to 80 percent for the Selenastrum capricornutum toxicity test.

B2.4 Test Materials for Soil and Sediment Samples

Tests should be conducted on soil and sediment samples as soon as possible after collection. The samples should be stored in the dark at 4°C. Prior to testing the soil and sediment samples are sieved through a 1/4-inch screen. The large pieces that will not pass through the screen are pulverized and rescreened. Rocks and debris are discarded. Soil and sediment samples for earthworm and seed germination tests are diluted with an artificial soil to the desired test concentration.

Soil or sediment (dry weight) sample quantities required to perform the following toxicity tests are:

Test Species	Soil Sample (g)
D. magna	175
S. capricornutum	125
L. sativa Seed Germination (soil)	1200
E. andrei Earthworm soil	1500

B2.4.1 Water Holding Capacity

The earthworm (E. andrei) seed germination toxicity tests require that the moisture content of the test soils be known. Furthermore, each toxicity test has its own requirement for moisture content at the beginning of a test. One must determine the water holding capacities of both the artificial and site soil so the appropriate moisture adjustments can be made. This is done by placing 100 grams dried test soil into a 250 mL glass beaker. Add 100 mL of deionized water to the soil. Mix thoroughly with a glass stir rod to ensure that all soil particles are wetted and that a slurry of soil and water exists. Fold a circle of 185 mm diameter, coarse porosity, qualitative crepe filter paper into quarters and place in a 100 mm (top inside diameter) by 95 mm (stem length) glass chemical funnel. The folded filter paper should be level with the top of the glass funnel. Slowly add 9 mL of deionized water, using a pipette, to the filter paper to wet the entire surface. Measure the combined weight of the funnel and hydrated filter paper. Add the 100 grams weight of the dried soil to this measured value to obtain the initial weight. For example:

Funnel and Hydrated Filter Weight = 163 grams
Weight of Dried Site Soil = 100 grams
Initial Weight = 263 grams

Place the funnel in a 500 mL glass erlenmeyer flask. Slowly pour the slurry of soil and water onto the hydrated filter paper held in the funnel. Rinse any soil remaining on the beaker and stir rod, into the funnel with deionized water. Use the minimum, but necessary, volume of water to assure that all of the solid material has been washed onto the filter. Cover the funnel tightly with aluminum foil and allow it to drain for three hours at room temperature. After three hours weigh the funnel, hydrated filter paper and soil slurry to obtain the final weight. The WHC equals the difference between the initial and final weight, expressed as mL water/100 g soil. For example:

Final Weight of
Funnel, Filter
and Soil Slurry = 302 grams
- Initial Weight = 263 grams
WATER HOLDING = 39 grams or 39 mL H_2O/gram soil
CAPACITY

B2.4.2 Hydration of Soils

Once the test soils are mixed, they need to be hydrated, based on the measured water holding capacity, with deionized water to create a moist, but not saturated, testing environment. For example, one wants a site soil hydrated at 80 percent of its WHC and the artificial soil to be hydrated to 80 percent of its WHC. If the site soil has an 80 percent WHC of 29.5 mL water/100 g soil and the artificial soil has an 80 percent WHC of 57 mL water/100 g soil, we would have the following situation with a 75 percent concentration of site soil:

(57.0 mL H_2O/100 g AS) (0.75) = 42.75 mL H_2O/100 g AS
(29.5 mL H_2O/100 g SS) (0.75) = 22.13 mL H_2O/100 g SS

These results define, on a site sample dry weight basis, the amount of water that must be added to the test soil to achieve an 80 percent WHC. However, the amount of water required to hydrate this test soil must to be reduced to compensate for the actual moisture content of the site soil. If the hypothetical site soil noted above had a moisture content of 0.20, a 100 gram aliquot of the site soil would contain (0.20 x 100 grams) 20 mLs of water. Therefore, the adjusted volume of water required for hydration of each 100 grams of site soil would be calculated as follows:

$$\frac{22.13 \text{ mL } H_2O}{100 \text{ g dry soil}} - \frac{20 \text{ mL } H_2O}{100 \text{ g wet soil}} = \frac{2.13 \text{ mL } H_2O}{100 \text{ g Site Soil}}$$

Each site sample concentration tested in the toxicity test must have the correct amount of hydration water calculated before the final additions of water are made to the test soils. For example, if 400 grams of site soil is to be mixed with 150 grams of artificial soil to make 550 grams of test soil, these weights are multiplied by the amount of hydration water needed:

$$\begin{array}{rl} (400 \text{ g SS})\ (2.13 \text{ mL } H_2O/100 \text{ g SS}) = & 8.52 \text{ mL} \\ \underline{(150 \text{ g AS})\ (42.75 \text{ mL } H_2O/100 \text{ g AS}) =} & \underline{64.13 \text{ mL}} \\ \text{Total} = & 72.65 \text{ mL} \end{array}$$

The total volume of 72.65 mL hydration water is then added to the 550 grams of test soil for the 75 percent concentration.

B2.4.3 Test Soil or Sediment pH Adjustment

The pH of the site soil should be measured prior to preparation of the test soil. The site soil pH should fall within the range of 5-9. Acid and basic site soils that fall outside this range must be adjusted to the closest pH, acceptable for survival of the test organisims, with either 5.0 N NaOH or 5.0 N HCl. A slurry composed of 2.5 mL deionized water per gram of site soil is mixed for 10-minutes with a spin bar on a magnetic stirrer before the pH is recorded. The amount of NaOH or HCl needed to adjust the site soil slurries to their allowable pH value is used to determine the quantity of caustic or acid added to the total required of the site soil. The NaOH or HCl is added during the hydration step discussed in Section A.2.4.2.

The pH can be adjusted when the test soil is hydrated using the information obtained from the pH measurement. If, for example, it was determined that 0.2 mL of 5.0N HCl was used to adjust 10 grams of site soil, then we would calculate the amount of HCl to be added for the above examples of 75% concentration using 3 replicates as follows:

(400 grams) (0.2 mL 5.0N HCl) = 8 mL 5.0N HCl
Site soil 10 grams

(132 mL H_2O) - (8 mL) = 124 mL H_2O
Artificial HCl
Soil

B3 *DAPHNIA MAGNA* 48-HOUR STATIC ACUTE TOXICITY TEST
(from Greene et al, 1988)

B3.1 Introduction and Rationale

The static toxicity test with freshwater macroinvertebrates uses less than or equal to 24-hour-old *D. magna*. The mean lethal concentration is calculated at 48 hours for *D. magna* (48-hour LC_{50}). When death is not easily detected, immobilization is considered equivalent to death.

B3.2 Materials and Methods

Procedures listed for all aquatic tests under GENERAL METHODS AND SAMPLE PREPARATION are applicable to the static acute toxicity tests with *D. magna*. Sections that should be followed are: Facilities, Cleaning and Preparation of Glassware, and Test Material. Materials and methods unique to *D. magna* tests are discussed below.

B3.2.1 Dilution Water

Dilution water can be reconstituted deionized, distilled, or tap water. The minimum criterion for acceptable dilution water is one in which healthy organisms survive for the duration of testing without showing signs of stress.

B3.2.2 Hardness Adjustment of Site Samples

Surface water, groundwater, soil elutriates, and sediment elutriates should be measured for hardness after they have been passed through the final 0.45 µm filtration step. The solution hardness is calculated for the highest site sample concentration that will be tested. If the calculated test sample hardness indicates that hardness in the sample will to fall below 80 mg/L $CaCO_3$ the site sample hardness should be raised to 100 mg/L $CaCO_3$ before preparing the test sample solutions.

The reagents listed below can be used to increase hardness of the test water as follows:

$$\text{DELTA TOTAL HARDNESS (DTH mg/L)} = \text{DESIRED HARDNESS} - \text{SAMPLE HARDNESS}$$

$$\text{MULTIPLICATIVE FACTOR (MF)} = \frac{[(\text{DTH mg/L})\ (\text{DESIRED VOLUME IN LITERS})]}{1000\ \text{mg/g}}$$

Multiply each of the following constants by the multiplicative factor, and add to the desired total sample volume in liters. Stir for at least 1 hour, or until dissolved.

$MgCl_2.6H_2O$	0.593
$CaSO_4.2H_2O$	1.219
$NaHCO_3$	1.230
$KHCO_3$	0.147

EXAMPLE: The test solution hardness is 55 mg/L $CaCO_3$.

$$DTH = 100 - 55 = 45 \text{ mg/L } CaCO_3$$

$$MF = \frac{[(45 \text{mg/L } CaCO_3)\ (2 \text{ L})]}{1000 \text{ mg/g}} = 0.09 \text{ grams}$$

The salts added 2 liters of solution to obtain a total hardness of 100 mg/L $CaCO_3$ are:

$CaSO_4 \cdot 2H_2O$ (1.219 x 0.09 MF) = 0.1097 grams
$MgCl_2 \cdot 6H_2O$ (0.593 x 0.09 MF) = 0.0534 grams
$NaHCO_3$ (1.230 x 0.09 MF) = 0.1107 grams
$KHCO_3$ (0.147 x 0.09 MF) = 0.0132 grams

B3.2.3 Species

D. magna (less than or equal to 24-hour old) are the species to be used in this freshwater static acute toxicity test. Daphnids occur in nearly all types of freshwater habitats.

B3.2.4 Source of Test Organisms

Daphnia magna should be reared in the testing facility from laboratory cultures.

B3.2.5 Size and Life Stages

Daphnia magna that are less than or equal to 24-hours-old should be used for testing. All organisms in a test must be from the same source and as healthy and uniform in size and age as possible.

B3.2.6 Culturing, Care, and Handling

Daphnia magna are maintained in a static system at 22± 2°C with a photoperiod of 16 hours light and 8 hours dark. Light intensity should be kept low to control unwanted excess growth of the live algae used to feed the daphnids. Water used to culture *D. magna* is not the same as that used for diluting site samples. Water from a "clean" local source (well, lake, or river) is used and reconstituted to a hardness of 200 mg/L $CaCO_3$ by following the method at the end of Section B3.2.2. The culture water should be of constant quality and analyzed by accepted methods to ascertain that none of the following substances exceeds the maximum allowable concentrations shown:

POLLUTANTS	MAXIMUM CONCENTRATION
Suspended solids	20 mg/L
Total organic carbon	10 mg/L
Unionized ammonia	20 ug/L
Residual chlorine	3 ug/L
Total organophosphorus pesticides	50 ng/l
Total organochlorine pesticides plus PCB's	50 ng/L

Cultures must be maintained under optimum conditions at all times to prevent formation of ephippial eggs. *D. magna* from cultures in which ephipia are being produced must not be used in testing.

Periodic subculturing, elimination of crowding, and adequate food will generally prevent problems in *D. magna* cultures. Twice each week (Tuesday and Friday) the adult daphnids should be screened and placed into fresh reconstituted hard water. Water should be added as necessary to maintain volume in the culture jars. Once the *D. magna* cultures are 4 weeks old they are discarded and replaced with new cultures inoculated with less than or equal to 24-hour-old organisms.

Water quality should be held constant as described above and temperature changes should not exceed 4°C in any 12 hour period.

Organisms should be handled as little as possible. When necessary, it should be done gently, carefully and quickly so that the organisms are not needlessly stressed. Wide-bore pipettes (0.5 cm) are best for handling D. magna. Organisms that touch dry surfaces or are injured during handling should be discarded. Test organisms should always be shielded from disturbances and overcrowding.

B3.2.7 Culture of S. capricornutum as a Food Source for Daphnia and Feeding Requirements

Daphnia are fed S. capricornutum once each working day. Monday through Thursday D. magna are fed from a stock culture of S. capricornutum in a quanitity sufficient to provide a final concentration of 2 mg/L dry weight (approximately 135,000 cells/mL) in the rearing jars. On Fridays the quantity is doubled to allow sufficient nutrients to sustain the cultures through the weekend without additional feeding.

Selenastrum capricornutum, cultured as a food source for D. magna, are incubated in an enviromental chamber held at 24±2°C with continuous light intensity of 4304±430 lux.

An algal nutrient solution is prepared with sterilized deionized water. The final volume is brought to 1 liter with filter-sterilized deionized water. Deionized water (not the culture medium) is filter-sterilized by passing through a 0.22 μm porosity cellulose membrane filter (pre-rinsed with 100 mL deionized water) into a sterile container. After the algal growth medium is prepared a final spike of 1 mL/L BME vitamin solution (Sigma Chemical Co.) is added.

The vitamin fortified algal growth media is used to fill a 4 liter aspirator bottle which has a serrated tubulature near the bottom. The tubulature is fitted with rubber tubing, for withdrawing algal cell suspensions, and clamped off. A magnetic stir bar is placed into the aspirator bottle and the bottle is place on a magnetic stirrer. Air from an aquarium pump is injected through glass tubing which was inserted in one of three holes drilled through a rubber stopper. The end of the glass tubing is located about 2 inches above the bottom of the aspirator bottle. The second hole is an air outlet. This outlet is fitted with Tygon tubing and the opposite end is placed in a 500 mL Erlenmeyer flask filled with 10 percent HCl. The HCl acts as an air lock to insure against outside contamination of the S. capricornutum culture. The third hole is an inlet for replenishing the nutrient solution after cultures have been tapped off for feed. When not in use the hole is capped. The cultures are discarded monthly. New cultures are started from sterile isolates of S. capricornutum.

D. magna are fed, in addition to the algal cells suspensions, a fish-food and yeast solution at the rate of 5 drops per 3 liter jar. This nutrient solution is fed (on Tuesdays and Fridays) after the water in the culture jars has been changed. The fish-food yeast formula is prepared by adding 12 grams fish-food (e.g., salmon or trout pellets) and 3 grams bakers yeast to 1 liter deionized or distilled water.

B3.2.8 Test Procedures

Unless approximate toxicity of the sample is already known, at least eight concentrations of test sample should be prepared. A series of test sample concentrations from 0 to 100 percent is recommended. A typical test might include, in addition to the 200 mg/L $CaCO_3$ reconstituted deionized water negative control, a test sample series (v:v percent) of 100, 80, 60, 30, 10, 6, 3, and 1.

The toxicity tests should be conducted at 22±2°C with photoperiod of 16 hours light and 8 hours dark. The organisms should not be fed during exposure. Test conditions are summarized in Table B-1. Daphnia magna tests are conducted with 10 organisms in 50 mL of test water in a 100 mL beaker. There are three replicates for each test concentration.

Toxicity test organisms should be placed in the test and control vessels not more than 30 minutes after the test solutions are prepared. Ten less than or equal to 24-hr-old D. magna are placed into each test vessel. Chemical, physical, and biological data are taken and recorded, for the duration of the test. Temperature, dissolved oxygen, pH and hardness should be recorded initially and at 48 hours on the high and low concentration. No further testing is required if toxicity is not detected at any concentration. The test material may be reported as having no detectable toxicity. Test materials that kill or immobilize nearly all test organisms at all dilutions should be retested with a lower dose range.

Mortality is used to define acute toxicity. Criteria for death are lack of movement and no reaction to gentle prodding. Mortality, immobilization, and abnormal behavior should be recorded. If greater than 10 percent of the control organisms die or are immobilized, the entire test is unacceptable.

Table B.1 Summary of Daphnia magna Test Conditions

TEMPERATURE	22 ± 2°C
PHOTOPERIOD, hours light:dark	16:8
WATER QUALITY, hardness (mg/L $CaCO_3$)	80
CONTAINER SIZE	100 mL
TEST VOLUME	50 mL
ORGANISMS PER CONTAINER	10
REPLICATES	3
FEED	No
DURATION, hours	48
MEASUREMENT OF DO	0, 48
MEASUREMENT OF pH	0, 48

B3.3 Results and Data Interpretation

The concentration of test material lethal to 50 percent of the population (LC_{50}), and 95 percent confidence limits should be determined at 48-hours for *D. magna* toxicity tests. Any of several methods including moving average, Spearman-Karber, Litchfield-Wilcoxin, probit, or binomial may be used.

B4 *SELENASTRUM CAPRICORNUTUM* 96-HOUR STATIC CHRONIC TOXICITY TEST (from Greene et al, 1988)

B4.1 Introduction and Rationale

Unicellular algae are important producers of oxygen and form the basis of the food web in aquatic ecosystems. Algal species and communities are sensitive to environmental changes and their growth may be inhibited or stimulated by the presence of pollutants. Therefore, the response of algae is an important consideration when assessing the potential ecological effects of industrial or municipal waste products entering surface and ground waters from hazardous waste sites.

A static chronic test for toxicity to algae can be conducted in 96-hours. Results of the toxicity test are expressed in terms of the EC_{50} (the lowest concentration causing inhibition of growth by 50 percent relative to the control). Growth less than the control indicates that the material is at least algistatic (inhibitory) under the conditions of the test. The cell culture can also be subcultured to determine if the test solutions are algicidal (lethal).

B4.2 Materials and Methods

General procedures listed for all aquatic tests in the GENERAL METHODS AND SAMPLE PREPARATION are applicable to the static chronic toxicity test with freshwater algae. Specific areas that should be followed are: facilities, construction materials, test material. Materials and methods unique to freshwater algal tests are included below.

B4.2.1 Equipment

Equipment must include a constant-temperature room or incubator capable of providing temperature control of 24 ± 2°C. A gyratory shaking apparatus capable of 100 oscillations per minute should be used for test culture flasks. Continuous illumination of 4304 lux (±10 percent) is required for *S. capricornutum*. Overhead cool-white fluorescent bulbs should be used. Light intensity is measured adjacent to the flask, at liquid level, using a light meter. An electronic particle counter with mean cell volume (MCV) computer, fluorometer capable of measuring chlorophyll *a*, or a general purpose microscope with a hemacytometer counting chamber are needed for measuring biomass.

B4.2.2 Test Containers

Algal tests should be conducted in 125-mL Erlenmeyer culture flasks (Pyrex or Kimax glass). The flasks should be stoppered with demonstrably non-toxic plugs. Do not use diSPo plugs (Scientific Products). They have been shown to release toxic constituents. All flasks should be cleaned and autoclaved for 20 min. at 1.1 kg/cm^2 (15 psi) and 121°C.

B4.2.3 Freshwater Algal Nutrient Medium

Algal Assay Medium (AAM) is prepared with filter-sterilized deionized water. The final volume is brought to 1 liter with filter-sterilized deionized water. Deionized water (not the AAM) is filter-sterilized by passing through a 0.22-µm porosity cellulose acetate membrane filter (pre-rinsed with 100 mL deionized water) into a sterile container. Medium should be constituted as needed, but can be stored for periods up to 1 month in the dark at 4°C to reduce possible photochemical changes and bacterial growth.

B4.2.4 Test Organisms and Culture Maintenance

For freshwater algal toxicity tests, the recommended test organism is *Selenastrum capricornutum*, a unicellular, non-motile chlorophyte that is easily mainitained in laboratory cultures. Obtain algal cultures (Culture No. ATCC 22662) from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 (phone, 1-800-638-6597).

Upon receipt of the algal culture, 1.0 mL should be aseptically transferred to AAM. The rest of the culture can be maintained up to 6 months in a dark refrigerator at 4°C. Weekly aseptic stock transfer is recommended to maintain a continuous supply of "healthy" cells for experimental work. To retain unialgal culture over a long period, it is advantageous to prepare a semi-solid medium by dissolving 1.0 percent agar in AAM. This medium is autoclaved, cooled to 45°C, and placed in sterile petri plates. A portion of a liquid algal culture is streaked onto the plate and incubated under standard conditions. Algae should be transferred onto fresh plates every 4 weeks. Fresh liquid cultures should be started by transfer of a single algal colony to liquid medium at 4-week intervals. Liquid cultures are used for test inoculation.

B4.2.5 Test Procedure (Bottle Method)

Full strength AAM is used to dilute site samples for the diluted test sample series. Dilution of site sample with AAM insures adequate nutrients for the algae, while avoiding chemical effects on nutrient inactivation. An 80 percent test solution (20 percent AAM) is the maximum permissible test concentration which ensures the presence of adequate nutrients. AAM NUTRIENTS ARE NOT SPIKED INTO THE TEST SOLUTION PRIOR TO ITS DILUTION WITH ALGAL ASSAY MEDIUM. The different concentrations of AAM in the test series is accounted for in the formula for calculating percent effect.

Fifty mL of test solution is dispensed into each of three replicate 125 mL Erlenmeyer flasks to obtain an eluate test series of logarithmic concentrations from 0 to 80 percent. A typical test might include, in addition to AAM negative control, a test solution series v:v (%) or w:v (%) of 80, 60, 30, 10, 6, 3, 1 and 0.1.

A 4 to 5 day stock culture is used as the inoculum source. Population density in the stock culture is determined by electronic particle counter or direct counting. The culture should always be checked microscopically to insure that it is unialgal and healthy.

Inoculum, which provides a concentration of 10,000 cells/mL in the test solutions at the start of the test, is aseptically added to each test flask. The volume of inoculum added should be between 0.1 and 1.0 mL. The test flasks are incubated for 96 hours in an environmental chamber held at 24 ± 2°C. Light intensity is maintained at 4304 lux (±10 percent). Free gas exchange through foam plugs is maintained throughout the test by continuous shaking of the test flasks at 100 rpm.

The pH of the test solution mixtures should be from 6 to 9. A pH outside this range can inhibit growth and confound the test results. Documentation of pH should consist of, at a minimum, measurement of the full strength test solution as well as the highest and lowest test sample concentrations at the start and finish of the test.

B4.2.6 Microplate Algal Toxicity Test (from Blaise, 1986)

Use of rigid polystyrene, U-bottomed, 96-well microplates (MP's) was made for microplating.

Fig. B.1 illustrates the technique used for MP toxicity tests. The same technique was used whether toxicity was determined by cell counts or ATP analysis. In the latter case, however, axenic cultures were utilized and work was performed in aseptic conditions until the incubation stage. Samples were processed as follows: approximately 10 mL were filter-sterilized through a membrane (Gelman, GA-8; 0.20μ) and serially diluted in sterile test tubes (16/160 mm) with demineralized water (Millipore Super Q system) which had been autoclaved (121 C, 1.1 $kg.cm^{-2}$ pressure). Dilutions were then poured one by one into a sterile plastic reservoir (Titertek reservoir, Flow Laboratories Inc.), and 200 μL aliquot parts were taken up and immediately transferred into the appropriate wells by means of multichannel pipette (Titertek, Flow Laboratories Inc.). This pipette was used to start all tests (4-hour and 4-day EC_{50}'s) with the exception of the first series of tests (8-day EC_{50}'s), when the Eppendorf repeating pipette was used. Algae and the AAM enrichment medium were added together in the same way to the MP wells in the following proportions: 10 μL of inoculum (1,000, 20,000 or 1,000,000 $algae.mL^{-1}$, respectively, for the 8-day, 4-day and 4-hour tests) and 10 μL of AAM concentrated 13.75 times so as to obtain a

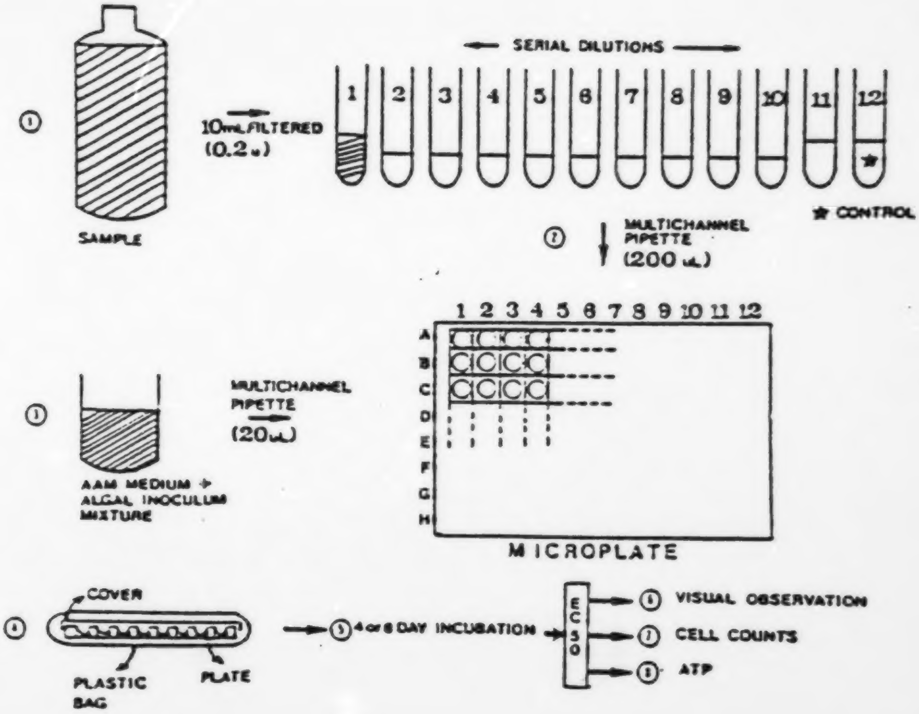

SERIAL DILUTIONS
①
SAMPLE
10mL FILTERED
(0.2μ)
1
2
3
4
5
6
7
8
9
10
11
12
★ CONTROL
②
MULTICHANNEL
PIPETTE
(200 μL)
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
MICROPLATE
③
AAM MEDIUM +
ALGAL INOCULUM
MIXTURE
MULTICHANNEL
PIPETTE
(20μL)
④
COVER
PLASTIC
BAG
PLATE
⑤ 4 or 8 DAY INCUBATION
EC50
⑥ VISUAL OBSERVATION
⑦ CELL COUNTS
⑧ ATP

Figure B.1 Microplate algal assay technique.

[†] total volumetric capacity per well is 300 μL.

theoretical biomass of 50 $mg.L^{-1}$ after 8 days of growth. The MP was then covered with its plastic lid and placed in a transparent bag (polyester film, 16.5 x 20 cm, Kapak Corp, St. Louis Park, MN, USA), whose open end was heat-sealed to prevent evaporation. Finally, the MP was placed in an incubator for the appropriate period in conditions identical to those described earlier for the classic test.

Several distribution patterns are possible for sample concentrations (and control) when conducting MP toxicity tests. The following method was chosed for industrial effluents (Fig. B.2): (a) 8 sample dilutions, each of which is replicated 6 times, spiked with AAM medium contributing a theoretical algal biomass of 50 $mg.L^{-1}$; (b) 8 sample dilutions, each of which is replicated 5 times, spiked with AAM medium without its P and N content: this addition does not contribute to algal biomass production per se but rather ensures a sufficiency in micro-elements such that the algal growth potential resulting from P and N utilization of the sample itself can be determined; (c) 8 control wells.

For the 4-day toxicity tests conducted, where the growth potential due to the sample was not taken into consideration, serial dilutions were arranged horizontally (Fig. B.1) and there were 8 replicates for each sample dilution and control (i.e., concentration X was placed in wells A1, B1,...H1, and the control was in wells A12, B12, ...H12).

At the end of the incubation period, MP well contents were re-suspended with a Rotatiter apparatus (Dynatech Products Inc.). For cellcount EC_{50} calculations, 100 µL were removed from each well with a micropipette and poured into an accuvette containing 9 mL of an electrolytic solution (Isoton, Coulter Electronics). Algae were then enumerated with the Coulter instrument. Identical volumes (100 µL) were also removed from each well for ATP EC_{50} determination. ATP was measured by bioluminescence using a previously described technique (Blaise et al., 1984).

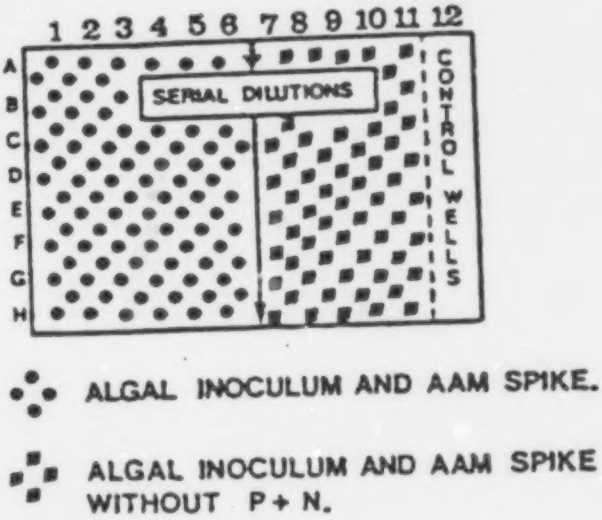

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
SERIAL DILUTIONS
CONTROL WELLS
ALGAL INOCULUM AND AAM SPIKE.
ALGAL INOCULUM AND AAM SPIKE WITHOUT P + N.

Figure B.2 Microplate Sample Distribution Pattern

To determine inhibition percentages, cell growth and ATP measured in sample concentrations were compared with corresponding control parameters. As indicated earlier and when applicable, it was necessary to take account of the biomass (B) resulting from N and P content of the sample (in the case of the spike without N & P), and of the biomass theoretically produced by the addition of the complete AAM spike (Bt). In both cases, generation of B and Bt depended on the toxicity present in the various sample concentrations. When calculating EC_{50}'s therefore, the biomass resulting exclusively from the potential growth of the sample (B) was deducted from that resulting from addition of the complete AAM spike (B + Bt). The ensuing biomass, Bt was compared with the Bt of the control for calculation of the EC_{50}. Just as for the classic test, the latter was either resolved graphically or reported as a toxicity range value when less than three inhibition percentages were available for plotting the toxicity line.

B4.2.7 Subculturing to Determine Lethality

A test solution should be subcultured into fresh AAM to assess whether the sample was lethal to, or inhibited, algal growth. Solutions are withdrawn from test flasks at the end of the 96-hour toxicity toxicity test. If the yield in the test flask is less than 25,000 cells/mL or less than 0.45 mg/L dry weight, 0.5 mL should be removed from each of the three replicate test flasks and added to one 500-mL flask containing 100 mL of fresh AAM. If the yield after 7 days is less than 25,000 cells/mL or less than 0.45 mg/L dry weight, the solution is algicidal (lethal). One must exercise care when subculturing to ensure that the 1.5 mL of test sample added to the 100 mL of AAM does not contain sufficient quantities of toxicant to produce algistasis. This source of error is greatest in those samples that produced EC_{50} values less than 10 percent.

B4.2.8 Response Monitoring

After 96-hours of exposure, algal growth is measured by any of the following methods:

- electronic particle counting;
- chlorophyll a fluorescence;
- microscopic counts; or
- biomass (gravimetric dry weight).

The algal toxicity test is designed to provide a comparative response to a large number of varying dilutions of sample. It is, therefore, better to use the rapid and more accurate electronic particle counter to measure growth.

B4.2.8.1 Electronic Particle Counting

An electronic particle counter with mean cell volume (MCV) computer is used. The particle counter offers the greatest precision and accuracy and is the preferred method if equipment is available and samples are suitable. The MCV computer must be calibrated with an organic calibration material.[1] Biomass may be determined directly by the following equation:

$$\begin{array}{c} \text{cell} \\ \text{counts} \\ \text{(cells/mL)} \end{array} \times \begin{array}{c} \text{mean cell} \\ \text{volume} \\ (\mu m^3) \end{array} \times \begin{array}{c} \text{conversion} \\ \text{factor**} \\ \end{array} = \begin{array}{c} \underline{S}.\ \underline{\text{capricornutum}} \\ \text{(mg/L dry weight)} \\ \end{array}$$

** Note that the conversion factor (e.g., 3.6×10^{-7}) may differ between laboratories and must be determined by each investigator.

Some test solutions form precipitates after sample preparation or during performance of the toxicity test. Laboratory technicians should be trained to recognize this problem and to make appropriate decisions on alternate bioassessment measurements when it occurs. If samples contain inordinate amounts of inorganic precipitates, an alternate means of response monitoring should be used. Since these occurrences are rare, it is reasonable to assess biomass fluorometrically by *in vivo* chlorophyll a measurement. The next best alternative is to assess biomass by direct microscopic counts. When choosing an alternative, one must determine whether particulate levels are great enough to interfere with light transmission or if the sample is colored to the extent that it would interfere with chorophyll a measurements. If this is a real concern, the microscopic cell counts are the only alternative.

---

[1] A 4.6 micrometer organic standard reference particle (part number 6602795), for calibrating the MCV, can be obtained from Coulter Electronics, Inc., Hialeah, Florida (phone 1-800-523-3713).

B4.2.8.2 Chlorophyll a Fluorescence

When precipitates form in the test solutions and interfere with electronic particle counting, chlorophyll a is the next best response measurement. The usefulness of chlorophyll a is sometimes limited, however, by color in the test solutions. Furthermore, some complex wastes may absorb or fluoresce in the same spectral region as chlorophyll a. Measurements of chlorophyll a fluorescence are made after first adjusting the fluorometer with a distilled water blank. The test solutions are well mixed by swirling the solution in the test flask. Six mL of solution is withdrawn and dispensed into the fluorometer chamber. After approximately 30-seconds, the relative fluorescence units (RFU) are recorded.

B4.2.8.3 Microscopic Counting

To determine cell numbers in the inoculum or create a standard curve for chlorophyll a direct microscopic counts are necessary. A hemacytometer counting chamber and a microscope are used. Two samples are taken from each flask, and two counts are made of each sample. Whenever feasible, 400 cells per replicate are counted to obtain ±10 percent accuracy at the 95 percent confidence level. Generally, this method is the least desirable for monitoring test response.

B.4.2.8.4 Gravimetric Dry Weight

The gravimetric dry weight method requires that a measured portion of algal suspension be filtered through a tared 0.6 µm PVC membrane filter. The filters are prepared as follows: dry for 2 hours at 70°C in an oven. Place filters in folded sheets of paper or aluminum weighing dishes on which the weights or codes are written. Cool in a desiccator for at least 1 hour and weigh. Filter a suitable portion of culture (50 mL or less as the cell density dictates) under a vacuum of 51 kPa. Rinse filter funnel with 50 mL distilled water using a wash bottle and let the rinsings pass through the filter. Dry at 70°C, cool in desiccator, and weigh. Subtract tare weight, divide by volume (liters) of culture filtered, and express as mg/L dry weight.

B4.3 Results and Data Interpretation

Percent inhibition (I) or stimulation (S) is calculated for each concentration according to the following formula:

$$\frac{(T\text{-}IN) - P(C\text{-}IN)}{P(C\text{-}IN)} \times 100 = \begin{array}{l} (-)\ \text{percent} = \text{Inhibition} \\ (+)\ \text{percent} = \text{Stimulation} \end{array}$$

Where:

P = percent volume of AAM used to dilute the test samples; must not be less than 20 percent;
C = maximum standing crop (mg/L) obtained in the AAM control;
T = maximum standing crop (mg/L) obtained in the test sample;
IN = dry weight (mg/L) of inoculum used at start of test.

For samples that are inhibitory, and EC_{50} (defined as the lowest test concentration inhibiting growth by 50 percent relative to the control) is calculated. The EC_{50} is calculated, for all samples, using linear regression analysis, by plotting the percent site sample concentration on the y-axis and percent effect on the x-axis.

B5 MICROTOX TOXICITY TEST PROCEDURE
(from Alberta Environmental Center Procedure)

The Standard Toxicity Test Procedure described was used by the participants in round-robins of the Microtox Interlaboratory Comparison Study (MICS) with the intent of maintaining uniformity and consistency.

B5.1 Apparatus and Reagents

- Microbics (Beckman) Microtox Model 2055 Toxicity Analyzer
- Chart Recorder
- Micropipettors: 10, 200, 500, 1000 µL
- Plastic Tips, disposable
- Refrigerator/Freezer
- Stopwatch
- Microtox Cuvettes
- Microtox Diluent
- Microtox Osmotic Adjustment Solution
- Microtox Reconstitution Solution
- Microtox Reagent, Lyophilized Bacteria (Photobacterium phosphoreum)

B5.2 Sample and Reagent Storage

B5.2.1 Samples

(a) Aqueous stock solutions (in deionized distilled water) of single chemical compounds will be freshly prepared.

(b) Samples will be supplied in clean, virgin, deionized distilled water washed screw-cap borosilicate glass tubes (16 x 125 mm) with Teflon coated liner screw caps.

(c) Refrigerate (4°C) samples prior to testing.

(d) DO NOT adjust sample pH as it is considered a part of the assay. Also, DO NOT modify or alter samples with any preservative or chemical agent. TEST ALL SAMPLES AS RECEIVED.

B5.2.2 Reagents

(a) The Microtox Reagent (lyophilized bacteria) will be stored in the freezer (-4°C to -10°C) of a nonself- defrosting refrigerator until required for analysis.

(b) Store the Microtox Diluent, Reconstitution Solution and Osmotic Adjustment Solution in a refrigerator (4°C). DO NOT FREEZE any of these reagents.

B5.3.0 Analyzer and Recorder Preparation

B5.3.1 Analyzer

(a) Check temperature readings of the analyzer on the digital panel meter (DPM), by depressing appropriate switches as follows.

Switch	DPM Temperature Reading °C
TURRET	15 ± 0.2
INCUB	15 ± 0.2
AIR	3 ± 1.0

(b) Depress the HV switch to turn on the voltage supply to the photomultiplier tube (PMI).

(c) Depress HV Check Switch, voltage reading on DPM should read between -500 and -900.

(d) Check PMT sensitivity by closing the turret (no cuvette) and adjusting SPAN (100% ADJ) at 4.0 turns (Clockwise) and the SENSITIVITY at 10x. Depress and hold CAL CHECK switch, the DPM reading should be between 080 - 120 if the analyzer is functioning properly. Reset SENSITIVITY to 1x.

(e) With the CAL CHECK still depressed, adjust the SPAN (100% ADJ) dial until the DPM reads 050. The recorder should agree (within ± 1% of scale) the DPM reading.

(f) Depress HV, RUN and SENSITIVITY 1x switches. The DPM should display 000 with the minus sign flashing. If necessary, unlock and readjust ZERO (ZERO % ADJ) dial, and relock to avoid accidental changes.

(g) If any of the above readings fall outside the specified range consult the manual for remedial action.

(h) Place new and clean cuvettes in the pre-cooling and all (15) incubator wells (3 horizontal rows of 5 wells designated as A, B, C and 1, 2, 3, 4, 5, respectively, see Figure B.3).

B5.3.2 Chart Recorder

(a) Turn the recorder on, remove cap from its pen, set chart speed to 1 cm/min, set input dial for 0-10 mV.

(b) With the analyzer DPM reading 000 (flashing minus sign), adjust the pen to the chart origin (zero) using recorder zero adjustment dial.

B5.4 Toxicity Test

NOTE: Before starting the assay, remove the Diluent and Osmotic Solution from the refrigerator.

B5.4.1 Reagent Reconstitution

(a) Remove reagent vial from the freezer to the refrigerator (4°C) and maintain for 20 to 30 minutes to allow temperature equilibration.

(b) Pipette 100 μL (1.0 mL) of the reconstitution solution into the pre-cooling well cuvette and allow to stand for 20 to 30 minutes for temperature stabilization.

(c) Take out one reagent vial from the refrigerator, remove and discard the aluminum seal and rubber stopper. When opening, make sure that the vial was under vacuum (listen for the vacuum releasing sound), and when open, the pellet is flat at the base of the vial. DO NOT USE A VIAL WITHOUT A VACUUM FOR TESTING.

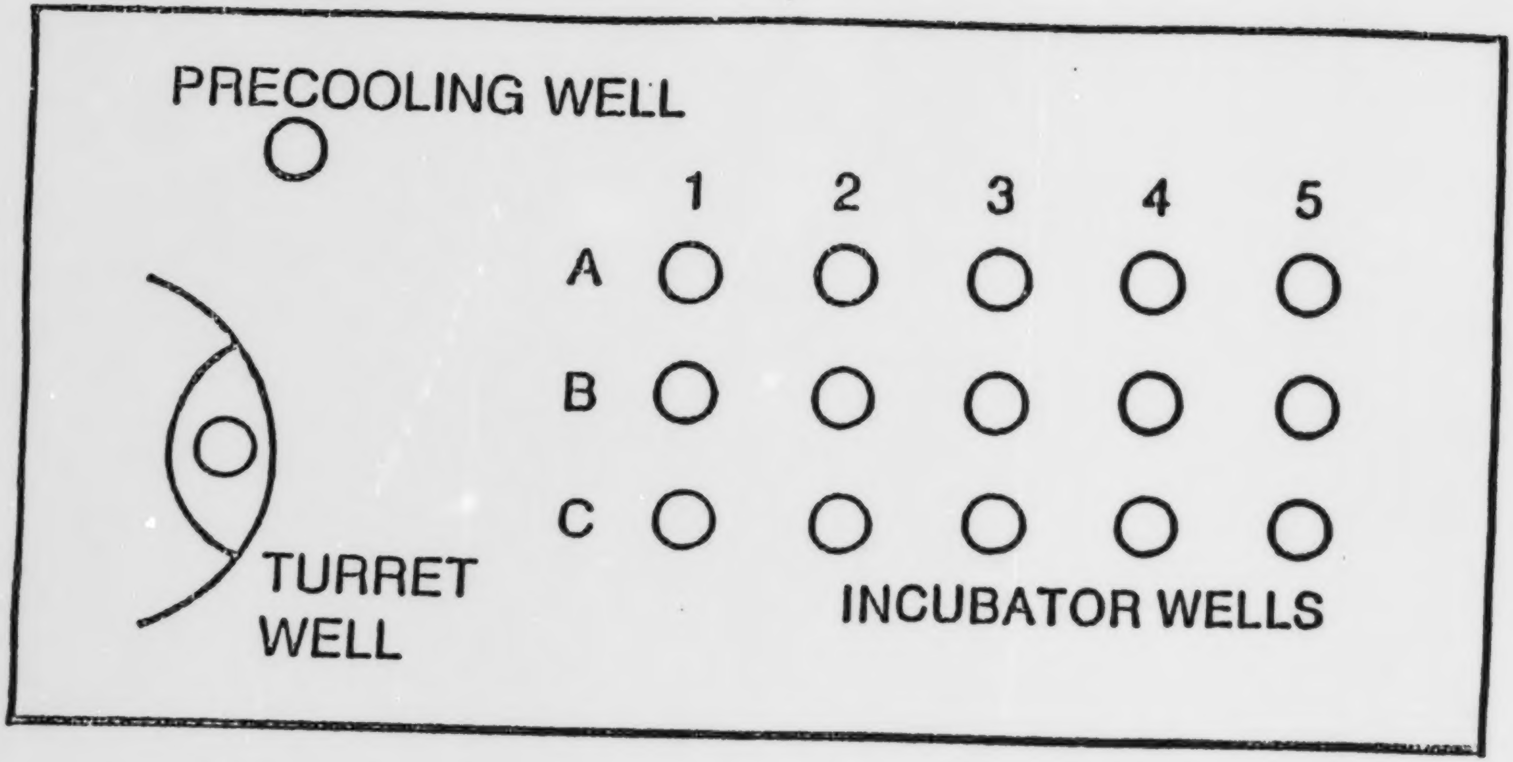

PRECOOLING WELL
1
2
3
4
5
A
B
C
TURRET
WELL
INCUBATOR WELLS

Figure B.3 INCUBATOR WELLS DESIGNATIONS

(d) Pour the pre-cooled reconstitution solution into the reagent vial by RAPIDLY inverting the cuvette. Mix by swirling for a few seconds while holding the vial at the top (to minimize warm-up).

(e) Pour back the reconstituted reagent into the cuvette and replace the cuvette into the pre-cooling well. Using a 500 µL micropipettor, immediately mix the reagent by aspirating and dispensing 500 µL of the solution about 5 to 10 times. Ensure to completely expel all liquid from the pipette tip during the last mixing. Maintain the cuvette in the pre-cooling well during the entire mixing step.

B5.4.2 Sample Preparation

NOTE: A two-fold serial dilution scheme will be used for the testing of all samples.

(a) Transfer 2000 µL (2.0 mL) of sample to the cuvette in well A1. Add 200 µL (0.2 mL) of Osmotic adjustment Solution. Using a 1000 µL micropipettor, mix well by carefully aspirating and dispensing the solution 3 to 5 times.

(b) Add 1000 µL (1.0 mL) of Microtox diluent to A2 through A5 and B1 cuvettes.

(c) Transfer 1000 µL (1.0 mL) of osmotically adjusted sample from A1 to A2. Mix the contents of A2 as in step _a_.

(d) Transfer 1000 µL (1.0 mL) of sample from A2 to A3. Mix the contents of A3 as in step _a_.

(e) Transfer 1000 µL (1.0 mL) of sample from A3 to A4. Mix the contents of A4 as in step _a_.

(f) Transfer 1000 µL (1.0 mL) of sample from A4 to A5. Mix the contents of A5 as in step _a_.

(g) Discard 1000 µL (1.0 mL) of sample from A5.

(h) Do not add any sample to B1 which will be used as non-toxic control (Reagent Blank).

(i) Allow 15 minutes for 15°C temperature equilibration. The sample concentrations at this point are:

Cuvette	Sample Concentration (%)
A1	90.9
A2	45.5
A3	22.7
A4	11.4
A5	5.7
B1 (Control)	00.0 (Reagent Blank)

B5.4.3 50% Assay

(a) Pipette 500 µL (0.5 mL) of Microtox diluent into cuvettes B2 through B5 and C1 through C5 (a total of 9 cuvettes).

(b) Transfer 10 µL (0.01 mL) of the reconstituted reagent (from the pre-cooling well) to cuvettes B2 through B5 and C1 through C5 (the same tip may be used for all transfers). Mix (3 to 5 times) by aspirating and dispensing with a 500 µL micropipettor.

(c) Allow 15 minutes for temperature equilibration. This can be done by using recorder set at a Chart Speed of 1 cm/minute and measuring the distance required for the 15 minute time period.

(d) After the equilibration period, check to ensure that HV, RUN and 1x SENSITIVIITY switches are in depressed position.

(e) Transfer the cuvette B2 into the turret, close the turret (opening handle in the READ position) and turn on the recorder. Adjust the recorder to read approximately 70-80% on the chart paper by adjusting the SPAN (100% ADJ) dial. Plot light level until a suitable slope (stable light output) is obtained.

(f) Adjust the SPAN (100% ADJ) dial for a DPM reading of approximately 090 (90% on chart recorder). Open the turret and remove cuvette B2 and return it to its incubator well.

(g) Cycle the cuvettes through turret in order B3, B4, B5, C5, C4, C3, C2, C1 to check for variability in light levels. If necessary the cuvettes may be reordered. Discard the three most deviant cuvettes (an acceptable light level is 080 to 100 on the DPM) leaving B2, B3, and B4 wells vacant.

(h) Note the setting on the SPAN (100% ADJ) dial at this time which indicates the health and stability of the reconstituted reagent. A setting of less than 5.0 turns of SPAN indicates healthy and relatively stable bacteria (a 090 DPM reading), whereas a setting of greater than 7.0 seems to indicate unstable and unhealthy bacteria. Repeat steps a to g using fresh diluent and reagents if setting exceeds 7.0.

(i) Cycle the cuvettes in the following order to obtain initial light (Io) levels for each cuvette: B5, C5, C4, C3, C2, C1. Allow 15-20 seconds between each reading.

(j) Immediately, and without removing the cuvettes from the incubator wells, transfer 500 µL (0.5 mL) of reagent blank (cuvette) B1 and sample dilutions (cuvettes A5 to A1) into cuvettes B5 and C5 to C1 as follows:
Pipette 500 µL from B1 to B5 (start the stopwatch at this point)
Pipette 500 µL from A5 to C5
Pipette 500 µL from A4 to C4
Pipette 500 µL from A3 to C3
Pipette 500 µL from A2 to C2
Pipette 500 µL from A1 to C1

Ensure that transfers are made by pipetting up the concentration gradient (i.e. order of increasing sample concentration) as specified above. Since the same sample has been serially diluted, use the same tip to transfer all sample dilutions. Mix each transfer thoroughly by aspirating and dispensing 500 µL volumes 3 to 5 times.

Also ensure that this challenge step is completed in the same amount of time in which the initial light measurements were made. A 15-20 seconds interval between each transfer is usually sufficient.

(k) Five minutes after the first challenge, cycle the cuvettes in the following order to obtain (I_5) light levels: B5, C5, C4, C3, C2, C1.

(i) Repeat the cycling of cuvettes for light readings at 10(I_{10}), 15(I_{15}) and 20(I_{20}) minutes as in step k.

(m) Since in the above procedure 500 µL (0.5 mL) of sample is mixed with 510 µL (0.51 mL) of diluted reagent, the final concentration of sample assayed is one-half of the prepared dilutions. Therefore, the final concentrations (of sample) in the test cuvettes for the designated serial dilution scheme and test procedure outlined above are:

Cuvette	Sample (Final Assay) Concentration (%)
B5	0.0 (Reagent Blank)
C5	2.8
C4	5.7
C3	11.4
C2	22.7
C1	45.4

Note that the final assay concentration for each cuvette and the light output readings I_0, I_5, I_{10}, I_{15}, and I_{20} are required for data reduction.

B5.5.0 Data Recording and Reduction

B5.5.1 Data Recording

(a) Record all details of the laboratory and sample information on the MICS "ASSAY DATA SHEET" (see Fig. B.4).

Figure B.4

MICROTOX INTERLABORATORY COMPARISON STUDY (MICS) - ASSAY DATA SHEET

LAB INFORMATION

LABORATORY NO. ____
ROUND-ROBIN NO. ____
ORGANIZATION ____
ADDRESS ____
AND ____
TELEPHONE NO. ____

SAMPLE INFORMATION

SAMPLE NO. ____
SAMPLE TYPE ____
STORAGE AT ____ °C
VISUAL COLOR ____
INITIAL pH ____
REMARKS ____

DATE PREPARED/SHIPPED ____
DATE RECEIVED ____
DURATION (AGE) ____ HRS
VISUAL TURBIDITY ____
OSMOTIC ADJUSTMENT ____ MOAS

OBSERVED AND CALCULATED RESULTS

REAGENT NO. ____
ASSAY DATE & TIME ____

ASSAY NO. ____
ASSAYED BY ____

COMMENTS

BLANK READINGS (CUVETTE B_1)	BI_0	BI_5	BI_{10}	BI_{15}	BI_{20}
	☐	☐	☐	☐	☐
SAMPLE (FINAL ASSAY) CONCENTRATIONS (ASCENDING ORDER)	____ CUVETTE C5	____ C4	____ C3	____ C2	____ C1
0 MIN READINGS	____	____	____	____	____
5 MIN READINGS	____	____	____	____	____
10 MIN READINGS	____	____	____	____	____
15 MIN READINGS	____	____	____	____	____
20 MIN READINGS	____	____	____	____	____

15 MIN-EC50 (% OF SAMPLE) ____

CALCULATION METHOD
__ GRAPH
__ CALCULATOR

PLETED BY

(b) Record the initial (I_o) and subsequent (final) I_5, I_{10}, I_{15}, and I_{20} light output readings, for the reagent blank and each assay sample dilution, on the MICS "ASSAY DATA SHEET". Preferably record light readings directly from the DPM during the assay. Alternatively, readings may be transcribed from the chart recorder after the assay is completed.

B5.5.2 Data Reduction

NOTE: For all calculations and data reduction, follow procedures detailed in the operating manual.

(a) Use Sharp Model EL-5100 Programmable Calculator or any "In-house" computer program for all calculations and data reduction as appropriate.

(b) Calculate and determine the assay endpoint (toxic responses) as the effective concentration of a test sample (toxicant) causing 50% reduction in light output (EC_{50}).

(c) Use Gamma (Γ) for all calculations and data reduction in place of percent light decrease (% Δ). Gamma (Γ) is the ratio of the light lost to the light remaining.

(d) Use the correction factor (Blank Ratio, BR) to accommodate the normal (time dependent) changes of light output by the bacteria in the absence of a toxicant. Determine BR by dividing the final time light intensity readings of the reagent blank (B_t) by the initial light intensity of the blank (B_o).

$$BR = \frac{B_t}{B_o}$$

$$\Gamma_{(t)} = \frac{\text{Corrected light lost}}{\text{light remaining}} = \frac{BR_{(t)}\ I_{(o)}}{I_{(t)}} - 1$$

(e) Calculate and determine EC_{50} values by the method (graphic or calculator) normally used in your laboratory. For graphical determination, plot Gamma (Γ) values as a function of sample concentration (C) on log-log graphic paper and determine EC_{50} intercept (Gamma = 1).

(f) Enter EC values (% of sample) for 15 minute exposure (15 MIN - EC50) on the "ASSAY DATA SHEET" for each assay. Indicate the method used for EC50 determination.

B5.6.0 Results Reporting

(a) Record and report results of each of the ten toxicity tests on the "ASSAY DATA SHEET". Use both sides of the same sheet for results of duplicate tests of each sample.

(b) Provide all raw data (light readings), and calculate and report the EC values for all toxicity tests.

B6 EISENIA ANDREI (Earthworm) 14-DAY SOIL ACUTE TOXICITY TEST (from Greene et al, 1988)

B6.1 Introduction and Rationale

Earthworms have been selected as an indicator species because they are representative of the terrestrial environment and are of considerable importance in improving soil aeration, drainage, and fertility. The soil test was chosen because the exposure conditions in this test closely resemble natural conditions.

Earthworms differ from aquatic organisms in that they may be exposed to toxic chemicals in the aqueous phase via soil moisture, in the vapor phase, or by direct contact with particulate matter on the surface of soil constituents. They may also be protected in soil because many chemicals become tightly absorbed onto soil fractions, particularly organic matter, and the soil colloids making up the clay fraction.

Eisenia andrei is the earthworm species chosen for use in this laboratory toxicity test.

B6.2 Materials and Methods

B6.2.1 Facilities

Facilities needed to perform this test include: (a) containers for culturing earthworms; (b) and environmental chamber for maintaining temperature of the cultures at 22 ± 2°C during culturing and testing; (c) test containers (1-pint canning jars); and (d) suitable balances to measure soil mixtures and sample weights. There should be a work area for placing the worms in the test vessels, fume hood, distilled water source, and refrigeration at 4°C available. The test facility must have a controlled environmental chamber capable of maintaining a uniform temperature at 20 ± 2°C.

B6.2.2 Glassware Preparation

The 1-pint jars used in the earthworm soil toxicity test are standard screw-top canning jars with lids and screw rings. A 1/16-inch hole is drilled or punched into the center of each lid. All jars should be cleaned before each test. The rinse cycle in some dishwashers is colder than the wash cycle; this creates a thermal shock which may crack the soft glass jars. The cleaned jars should be dried at 50°C in a forced air oven and stored with lids (without holes) on, in a dust-free environment until needed. The lids should be examined after each use because the seal may deteriorate during repeated washing. Replace the lids and screw rings as needed.

B6.2.3 Test Species

The species identity of the test organism should be verified using appropriate systematic keys. Adult *E. andrei* (at least 2 months old with a clitellum) used for each test should weigh 300-500 mg and be from the same culture container.

Eisenia andrei is easily cultured, reaching maturity in 7- to 8-weeks at 20-25 degrees C. It is a hardy laboratory species, yet it is relatively responsive to various toxicants. Adult worms (at least 2 months old with a clitellum) are used for each test. They should weigh 300 to 500 mg and be from the same culture container.

B6.2.4 Earthworm Culture and Maintenance

Eisenia andrei stock cultures are maintained in a growth chamber at room temperature (20-20°C) in a rectangular container measuring about 24 x 15 x 17 cm. A mixture of alfalfa pellets saturated with water and aged for 2-weeks is used as the maintenance organic food substrate for the worms. The aged food is sprinkled on the surface of the culture tray. The worms are grown in peat moss bedding, pH adjusted between 5 and 8 by adding up to 3 percent by weight of $CaCO_3$.

Crowding of adult worms in the growth container must be avoided. Crowding decreases the growth rate and reproduction efficiency. Splitting the bedding material in half every 3-4 months with pH adjusted peat moss will prevent overcrowding.

Water is added when the bedding appears dry on the surface; however, saturation can cause the earthworms to crawl out of the rearing container. Water should be added at a rate that keeps the material wet, but avoids standing water in the bottom of the rearing pan. Water use will depend upon the ambient temperature and relative humidity. However, watering is easily adjusted to the particular conditions of the rearing room. The range of pH between 5 and 8 is best for optimum growing conditions. Worms subjected to moisture and pH conditions outside the optimum range will leave the container or be killed.

B6.2.5 Preparation of Test Soil

Homogenized waste site soil is mixed with artificial soil to prepare geometric test soil concentrations (i.e., 80, 40, 20, 10, 5, and 0 percent dry weight/dry weight). To ensure even distribution of the test soil mixture, the total amount for each concentration is mixed together before dividing into replicates.

The artificial soil consists of, by weight, 10 percent 2.36 mm screened sphagnum peat, 20 percent colloidal kaolinite clay, and 70 percent grade 70 silica sand. The final pH of the artificial soil is adjusted to 6.5 ± 0.5 with pulverized calcium carbonate, which is added to the mixture at usually less than 1 percent of the total.

The pH of the site soil should be measured prior to preparation of the test soil. The site soil pH should fall within the range of 5-9. Acidic and basic site soil that falls outside this range must be adjusted to the closest allowable pH with either 5.0 N NaOH or 5.0 N HCl. Test soil mixtures are based on the ratio of site soil to artificial soil used for each level of exposure. For example, if a 80 percent concentration with 3 replicates of 200 g each is desired, then we have;

$$660 \text{ g SS} \times 0.80 = 528 \text{ g SS} + 132 \text{ g AS}$$

The additional 60 grams of test soil prepared for each concentration is to allow for soil lost during preparation and dispensing (container walls, etc.). Once the test soils are mixed, they need to be hydrated with distilled or deionized water based on the water holding capacity to create a moist, but not saturated, testing environment. Both the site soil and the artificial soil are hydrated at 75 percent of their water holding capacity.

B6.2.6 Toxicity Test Procedure

Earthworms should be harvested and purged of their gut content for 24-hours prior to testing. Purging is done by placing 30-40 worms on a wetted (10 mL) G17 grade filter paper that has been placed in a 150 mm plastic petri dish. The lid is placed on the petri dish and worms are incubated at 20 ± 2°C under continuous light (400 to 800 lux). The average weight for purged worms is measured at the start of the test. They must weigh between 300-500 mg. Sufficient numbers of worms should be sorted to ensure that healthy individuals of the proper weight are available for the test. Animals that appear to be dead or injured must be discarded.

Ten (10) earthworms are placed into each of the three replicate jars which each contain 200 g (dry weight) of test soil. Adult earthworms can be handled without damaging them if care is exercised. The worms should be placed on the surface of the test soil in a pint jar, capped, and secured. The earthworms are not fed during the test period.

The pint test jars are incubated at 20 ± 2°C under continuous light to encourage the earthworms to burrow into the test soil. The test jars must not be shaded. Estimation of an LC_{50} toxicity test will require three replicates of a control and at least five test soil concentrations chosen in a geometric series. A typical test might include, in addition to the negative control, a test soil series (w:w percent) of 80, 40, 20, 10, and 5 percent site soil or sediment. Mortality assessment is obtained by emptying the test soil onto a tray and sorting the worms from the soil. Dead worms are discarded. Live worms are placed back into their test jars and placed on the surface of the soil. Decay of dead worms can be rapid and, if all 10 worms per container are not found, it can be assumed that the worms decayed beyond recognition.

B6.3 Results and Data Interpretation

The dead earthworms should be counted, for each exposure level, after 7-days and 14-days exposure. Test concentration (percentage of site soil) mortality, dose-response curves should be calculated at the end of the 7-days and 14-days exposure and are expressed as an LC_{50} endpoint.

B7 SEED GERMINATION 120-HOUR SOIL CHRONIC TOXICITY TEST

(from Greene et al, 1988)

B7.1 Introduction and Rationale

In the germination test, seeds are exposed to total available toxic constituents in the soil.

B7.2 Materials and Methods

B7.2.1 Facilities

The facilities must include work areas for placing the seeds into the petri dishes. There should be a fume hood, deionized or distilled water source, and refrigeration at 4°C available. The test facility must have a controlled environmental chamber capable of maintaining a uniform temperature of 24 ± 2°C and 4304 ± 430 lux of light operating on a clock timer to control diurnal cycling.

B7.2.2 Test Containers

The bottom half of plastic petri dishes, 150-mm (W) by 15-mm (H) are used for planting seeds.

B7.2.3 Equipment and Supplies

Items specifically needed include forceps, pH meter, storage bottles, 150-mm and 15-mm plastic disposable petri dishes, and 12" x 12" polyethylene Ziploc bags. An illuminated magnifier may be helpful for seedling examination.

B7.2.4 Test Organism

Seeds of the selected species should germinate, grow rapidly, contain no natural inhibitors, and require no special pretreatment.

The seeds used in this test are available from commercial seed companies. Seed from one lot for each species should be purchased in amounts adequate for 1 year's testing. Information on seed lot, year, or growing season collected and germination percentage should be provided by the source of seed. Only untreated (not treated with fungicide, repellents, etc.) seed is acceptable for use in this toxicity test.

B7.2.5 Size Grading of Seeds

After purchase, size grading is carried out on the entire seed lot for each species. Small samples, 100-150 grams, are sized at a time. The seed lot must be inspected. Trash, empty hulls, and damaged seed are removed. Depending on species, select series of four screens to separate sample size classes. The four screens are nested with the screen containing the largest holes on top and screens with successively smaller holes in sequence below. A blank or bottom pan collects the fraction that passes through all screens. Seed is poured onto the top screen and the whole set of nested screens are shaken (by hand or with a vibrator) until all the seed remains on one screen or reaches the bottom pan. The separated fractions are set aside and saved. This procedure is repeated until all the seed in the lot is sized. The size class containing the most seed is selected and used for the duration of the tests. The seeds in each sized class are divided into small lots, placed in separate envelopes or sacks, and stored in moisture-sealed containers in a refrigerator at 4°C.

B7.2.6 Preparation of Glassware

The petri dishes and Ziploc bags are disposable. However, all glassware used in preparing the soil samples must be thoroughly washed.

B7.2.7 Toxicity Test Procedure

Estimation of an EC_{50} toxicity test will require three replicates of a control and at least five test soil concentrations chosen in a geometric series. A typical test might include, in addition to the negative control, a test soil series (w:w percent) of 80, 40, 20, 10, and 5 percent site soil or sediment.

Each 150-mm plastic petri dish, containing 100 g of air-dried control or test soil, is seeded with 40 seeds. Approximately 90 g of washed 16-mesh silica sand is placed in an even layer over the top of the seeds and irrigated to 85 percent water holding capacity. Samples are prepared in triplicate, for each test soil and negative control (20 mesh washed silica sand = artificial soil). Each petri dish is placed into a polyethylene Ziploc bag and icubated at 24 ± 2°C in the dark for 48-hours, followed by 16-hours of light and 8-hours darkness until termination of the test at the end of 120-hours. Percent germination is determined for the controls and treated samples.

B7.2.7.1 Artificial Soil (AS)

Commercially available 20-mesh washed silica sand. The artificial soil is used as the negative control and for preparation of test soil mixtures (weight:weight) of artificial soil and site soil (SS).

B7.2.7.2 Cover Sand (CS)

Commercially available 16-mesh silica sand which has been passed through a 20-mesh sieve to remove fines. The cover sand is used to cover the planted test soils prior to adjustment of the water holding capacity and incubation of the test.

B7.2.7.3 Procedure for Planting Seeds

Forty seeds are distributed with a forceps over the surface of the test soil (TS). The seeds should be spaced at least 1/2" from the edge of the petri dish to ensure that they will be covered with test soil. The seeds are pressed into the surface of the test soil with the bottom of a clean beaker.

Distilled or deionized water is added very slowly with a capillary pipette over the seeds and test soil to attain 85 percent of its water holding capacity (WHC). The WHC of the site soil and artificial soil is determined. The WHC of the artificial soil (20-mesh sand) is approximately 19 mL/100 g. If a site soil has a WHC of 8.0 mL/100g, the corresponding volume of water (mL) to be added to a 50 percent test soil mixture (50 percent AS and 50 percent SS) would be 11.5 mL/100 g [e.g., 0.85 x ((0.5 x 19) + (0.5 x 8)) x 0.85 = mL hydration water to achieve 85 percent WHC].

Cover sand (90 g) is poured over the top of the hydrated test soil in a uniform layer. Final leveling can be done with a ruler or small piece of cardboard. Petri dish covers are placed over the planted test soil to prevent water loss and spillage of sample prior to incubation.

B7.2.7.4 Incubation

The covered petri dish is placed into a Ziploc plastic bag and centered over the bottom of the bag. The sides of the bag are raised into a vertical position over the dish. The dish cover is removed and the bag is sealed, leaving as much air space as possible. The closure should be airtight. The sealed bags are placed randomly throughout the growth chamber and incubated at 24 ± 2°C for 120-hours. The first 48-hr of incubation are in darkness. The remaining 72 hr are in a 16-hr light:8-hr dark diurnal cycle. The light intensity should be 4304 ± 430 lux.

B7.2.7.5 Measurement of Germination

After 120-hours, the number of germinated seeds in each dish is determined by counting each seedling that protrudes above the soil surface.

B7.3 Results and Data Interpretation

Specific criteria must be met for each of the test species to accurately estimate the EC_{50}. At least 80 percent of the seeds in the control dishes must germinate.

APPENDIX C

RAW TOXICITY TEST DATA AND CONFIDENCE LIMITS

TABLE C.1

RESULTS FOR DAPHNIA MAGNA

LC_{50} (%)
[Confidence Interval]

Company	Sample A #1	Sample A #2	Sample A #3	Sample B #1	Sample B #2	Sample B #3	Sample C #1	Sample C #2	Sample C #3
	0.0027 [0.0011-0.0088]	0.0031 [0.0016-0.0104]	0.0022 [0.0010-0.0077]	21.81 [19.55 - 24.33]	16.28 [13.98 - 18.97]	16.40 [13.99 - 19.23]	0.33 [0.16 - 0.66]	0.34 [0.27 - 0.42]	0.40 [0.30 - 0.52]
BIOQUEST	0.0025 [0.0010-0.0076]	0.0026 [0.0012-0.0081]	0.0026 [0.0011-0.0063]	20.31 [18.10 - 22.78]	16.61 [14.50 - 19.00]	17.13 [14.98 - 19.60]	0.75 [0.46 - 1.23]	0.45 [0.33 - 0.62]	0.38 [0.28 - 0.52]
	0.0021 [0.0008-0.0087]	0.0028 [0.0015-0.0077]	0.0019 [0.0008-0.0075]	16.70 [11.72 - 23.78]	18.00 [15.69 - 20.66]	16.53 [14.43 - 18.92]	0.91 [0.56 - 1.49]	0.46 [0.34 - 0.63]	0.45 [0.33 - 0.60]
EC (Edm)	0.72 [0.56 - 0.93]	0.67 [0.49 - 0.91]	0.71 [0.57 - 0.89]	37.8 [35.9 - 39.7]	33.5 [31.9 - 35.1]	32.5 [30.9 - 34.3]	17.4 [15.6 - 19.4]	11.9 [11.3 - 12.6]	11.4 [10.5 - 12.4]
EPA	2.33 [1.88 - 2.89]	2.50 [2.01 - 3.11]	2.18 [1.78 - 2.66]	34.82 [28.48 - 42.57]	37.32 [30.11 - 46.26]	-	0.17 [0.13 - 0.21]	0.13 [0.10 - 0.16]	0.16 [0.13 - 0.19]

TABLE C.2

ALGAE (Selenastrum capricornutum) RESULTS
EC_{50} (%)
[Confidence Interval]

Company	Sample A			Sample B			Sample C		
	#1	#2	#3	#1	#2	#3	#1	#2	#3
EC (Edm)	0.84 [0.44-1.25]	1.12 [0.96-1.29]	0.60 [0.06-1.13]	37.18 [29.10-45.27]	18.64 [0.33-36.96]	33.66 [10.35-56.97]	30.35 [16.96-43.74]	21.79 [9.94-37.64]	15.80 [5.60-26.00]
EPA	0.21 [0.16 - 0.25]				25.20 [20.00 - 45.00]			12.30 [7.50 - 16.50]	

TABLE C.3
RESULTS FOR MICROTOX
EC (%)
(Confidence Interval)

	EC_{20} (5 min)			EC_{20} (15 min)			EC_{50} (5 min)			EC_{50} (15 min)		
Company	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
#1	0.082 [0.062-0.107]	5.378 [3.736-7.743]	3.891 [3.389-4.468]	0.080 [0.071-0.092]	5.260 [3.699-7.479]	3.843 [3.239-4.559]	0.302 [0.209-0.435]	22.738 [15.936-32.442]	21.720 [19.418-24.295]	0.298 [0.252-0.353]	26.108 [18.145-37.565]	23.649 [20.489-27.297]
BETA	0.610 [0.562-0.663]	3.497 [3.194-3.828]	5.438 [4.687-6.309]	0.520 [0.464-0.584]	2.862 [2.503-3.272]	5.271 [4.770-5.826]	1.70 [1.576-1.833]	20.5 [19.187-21.976]	22.716 [19.636-26.279]	1.514 [1.379-1.663]	20.80 [18.983-22.791]	25.133 [22.708-27.816]
#3	0.343 [0.289-0.406]	5.372 [4.504-6.408]	5.970 [4.540-7.850]	0.205 [0.179-0.234]	4.924 [4.362-5.560]	5.571 [4.495-6.904]	1.172 [0.970-1.415]	26.615 [22.102-32.049]	22.825 [17.227-30.243]	1.126 [1.004-1.263]	29.269 [25.738-33.284]	24.717 [19.798-30.859]
#1	0.5	38.6	0.0	0.1	28.9	0.0	1.5	83.1	0.1	0.5	103.4	0.0
ESSO	0.5	36.8	4.3	0.4	26.0	4.3	1.3	97.3	19.0	0.9	138.0	19.4
#3	0.5	8.4	0.7	0.5	6.2	0.9	1.4	33.5	4.0	1.1	30.7	3.5
#1	1.25 [0.35-4.46]	7.43 [5.30-10.41]	0.94 [0.05-16.44]	0.34 [0.16-0.69]	6.15 [3.22-11.73]	0.63 [0.03-14.56]	2.59 [0.99-6.82]	36.94 [30.52-44.70]	8.35 [2.82-24.67]	0.85 [0.48-1.50]	33.64 [25.06-45.15]	7.38 [2.30-23.63]
ERCB	0.6 [0.13-2.80]	10.53 [8.45-13.13]	2.80 [1.49-5.24]	0.31 [0.04-2.28]	6.18 [4.43-8.60]	2.91 [2.02-4.20]	1.56 [0.49-4.92]	44.78 [36.90-54.35]	21.43 [18.22-25.21]	0.83 [0.18-3.91]	43.31 [35.79-52.42]	23.5 [21.35-25.87]
#3	0.89 [0.14-5.66]	10.55 [7.30-15.25]	2.99 [1.90-4.71]	0.17 [0.02-1.22]	8.03 [4.54-14.20]	3.12 [2.02-4.82]	2.00 [0.49-8.17]	47.59 [33.84-66.92]	17.27 [15.08-19.79]	0.51 [0.11-2.37]	50.58 [32.33-79.13]	18.87 [16.65-21.39]

TABLE C.3

RESULTS FOR MICROTOX (concluded)
EC (%)
(Confidence Interval)

	EC_{20} (5 min)			EC_{20} (15 min)			EC_{50} (5 min)			EC_{50} (15 min)		
Company	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
#1	-	-	-	-	-		0.19 [0.14-0.26]	18.6 [16.1-21.6]	17.9 [16.2-19.7]	0.19 [0.15-0.24]	20.7 [17.0-25.2]	17.4 [14.4-21.13]
EC (Edm)	-	-	-	-	-		0.23 [0.18-0.30]	17.6 [11.2-27.6]	20.2 [17.2-23.6]	0.23 [0.17-0.31]	20.6 [17.5-24.2]	21.4 [15.0-30.5]
#3	-	-	-	-	-		0.20 [0.15-0.27]	18.5 [14.8-23.0]	14.2 [12.0-16.8]	0.22 [0.15-0.33]	20.7 [15.7-27.3]	17.7 [16.4-19.0]

TABLE C.4

RESULTS FOR EISENIA ANDREI

EC_{50} (%)
[Confidence Interval]

Company	Sample A	Sample B			Sample C		
EPA	LT 0.63 -	3.30 [2.88 - 3.78]	3.49 CI not reliable	2.87 [2.35 - 3.51]	25.94 CI not reliable	26.39 [23.14 - 30.10]	24.62 [20.66-29.34]
BIOQUEST	LT 0.6 -	2.17 [1.54 - 3.06]	3.56 [2.58 - 4.92]	3.30 [2.36 - 4.59]		GT 10.0 -	

TABLE C.5

SEED GERMINATION RESULTS

EC_{50} (%)
(Confidence Interval)

Cucumber	Sample A #1	Sample A #2	Sample A #3	Sample B #1	Sample B #2	Sample B #3	Sample C #1	Sample C #2	Sample C #3
EPA	7.30 [6.45-8.29]	6.33 [5.41-7.41]	6.89 [6.21-7.76]	17.99 [13.91-23.26]	23.53 [19.90-27.83]	13.82 [11.58-16.50]	5.90 [5.65-6.16]	9.18 [8.49-9.92]	8.54 [8.14-8.96]
AEC	7.86 [7.08-8.72]	8.55 [7.93-9.22]	9.24 [8.71-9.80]	34.09 [31.35-37.06]	43.28 [37.60-49.81]	32.88 [30.11-35.91]	33.16 [27.74-39.65]	15.10 [7.29-31.28]	16.13 [3.03-85.90]
BETA	2.10 [1.44-3.08]	3.70 [2.23-6.14]	1.00 CI not reliable	26.91 [20.80-34.80]	27.85 [22.92-33.85]	35.75 [33.56-38.09]	37.39 [34.95-40.00]	-	-
Barley	**Sample A #1**	**Sample A #2**	**Sample A #3**	**Sample B #1**	**Sample B #2**	**Sample B #3**	**Sample C #1**	**Sample C #2**	**Sample C #3**
EPA	4.07 [3.65-4.54]	3.78 [3.39-4.21]	3.57 [3.19-3.98]	19.18 [15.90-23.13]	16.62 [14.74-18.74]	21.22 [18.10-24.88]	14.78 [12.00-18.20]	29.73 [23.13-38.22]	23.44 [18.90-29.07]
AEC	3.60 [2.74-4.74]	5.68 [4.11-7.86]	4.70 [3.73-5.92]	26.62 [20.84-34.01]	33.34 CI no reliable	25.85 [21.20-31.53]	19.55 [16.17-23.63]	23.60 [15.19-36.65]	15.55 [11.91-20.30]
BETA		LT 1.00 (couldn't calc.)		3.47 [2.71-4.46]	3.70 [3.14-4.37]	3.36 [2.68-4.20]	35.36 [33.02-37.86]	35.36 [32.67-38.26]	37.29 [33.95-40.96]